LOCKHEED CALIFORNIA COMPANY
Division of Lockheed Aircraft Corporation
BURBANK CALIFORNIA

Investigation of the Effects of Ionizing Radiation on the Central Nervous System in Vivo and in Vitro

PHYSICAL AND LIFE SCIENCES LABORATORY

INVESTIGATION OF THE EFFECTS OF IONIZING RADIATION ON THE CENTRAL NERVOUS SYSTEM IN VIVO AND IN VITRO

Contract NASw - 787

By

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FOREWORD

This is the year-end report for the first research year from July 19, 1963 to July 19, 1964 under Contract No. NASw - 787.

During the first research year a significant portion of the effort was necessarily devoted to technological preparations and developments. Further research in this area would be rewarding in its yield of other significant experimental findings.

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I. INTRODUCTION

For many years the adult nervous system was thought to be a relatively radioresistant organ. More recent studies, however, indicate that low doses of ionizing irradiation may cause subtle changes in the brain. In view of numerous reports claiming low dose effects, further thorough studies of the effects of ionizing irradiation on electrical changes in the central nervous system were proposed.

Observations of changes of the cerebral cortical activity (EEG) and of pools of neurons and neuroglial cells (depth recording) were begun using animals with chronically implanted brain electrodes. The susceptibility to ionizing irradiation of different brain cell constituents can probably best be examined in vitro by the tissue culture method which allows physiological examination of living single cells under visual control. Such studies were begun.

II. THE EFFECTS OF IONIZING RADIATION ON THE CENTRAL NERVOUS SYSTEM IN X-IRRADIATED CATS.

1. Pertinent Literature

The recording of electrical activity in the central nervous system of warm-blooded animals following ionizing radiation has been limited almost exclusively to studies of EEG patterns and of changes in post-radiation convulsive thresholds.

Slowing of the electroencephalogram in monkeys exposed to total-body X-irradiation was reported by Eldred and Trowbridge (9). Ross, Leavitt, Holst, and Clemente (35) studied post-irradiation EEG changes in a series of 37 monkeys exposed to head X-irradiation ranging from 6,000 r down to 1,500 r. They observed early slowing of EEG activity (3 - 5 per second), with an increase in wave amplitude. Animals exposed to 1,500r gradually developed focal abnormalities in the form of short bursts of high amplitude waves. Slowing of the EEG was also reported by Brooks (4) and Rube (36) who observed this effect in guinea pigs one to three days following exposure to doses over 2,000 r. On the other hand, Russian investigators (20;28) have claimed EEG alterations following doses of under 25 r.

A reduction of the convulsive threshold has been described by Andrews (2) who used high doses (10 Kr) in guinea pigs. A similar effect at lower doses (500 r) was reported by Schwartzbaum et al. (38). The most remarkable reduction in convulsive threshold was described by Miller (30) who claimed a significant susceptibility of DBA mice to audiogenic seizures following chronic exposure to gamma radiation at a total dose as low as 0.14 r.

Conditioning effects at lower dose rates have been reported by Garcia et al. (16; 17), Hug (24), and recently by Hunt and Kimeldorf (25).

Other electrophysiological studies than EEG tracings were carried out by Lee et al.(29; 46) who observed amplitude reductions of the evoked cerebral cortical and cerebellar responses in adult cats following X-irradiation. Variations in postsynaptic induced visual cortical potentials following 400 to 1,600 r were described by Etienne and Posternak (10). Gangloff and Haley (14; 15) have reported changes of spontaneous and evoked brain electrical activity in cats following 200 to 400 r. Also of interest is the fact that these investigators and Monnier and Krupp (31) have found an increased hippocampal discharge following head irradiation.

EEG patterns and behavioral effects from X-irradiation of the hippocampal system have been discussed recently by Schoenbrun, Campeau, and Adey (37). These investigators also reported large impedance baseline excursions in the hippocampus after focal irradiation.

Radiation damage to neuroglial, neuronal, and endothelial elements of the intact central nervous system has been shown by several investigators (Campbell and Novick (5); Clemente and Holst (6)), and the fact that the central nervous system is not a radioresistant organ now seems well established (Haley and Snider (21). The degree of radioresponsiveness and the pathogenesis of brain radiation damage, however, are still to be established.

2. Methods and Materials

Experimental Animals Used. The effects of ionizing radiation on the central nervous system were studied in X-irradiated cats with chronically implanted brain electrodes. The animals were prepared under barbiturate anesthesia. Figures 1 through 4 demonstrate essential phases of the operation. The head of the animal was properly positioned in a stereotaxic instrument. Parts of the skull were exposed and freed from periosteum. Accurately localized trepanation was carried out, using a small drill (Fig. 1). With the aid of the stereotaxic instrument, recording and stimulating electrodes of the bipolar strut type were inserted into various deep areas of the brain, including the hippocampus, basal forebrain regions, reticular formation, thalamic nuclei, and the amygdala. The exact position of the electrodes was predetermined by stereotaxic co-ordinates (Jasper, Ajmone-Marsan, 1954). When the electrodes were inserted into hippocampal areas, the position of the electrode tip could be verified during the process of implantation by recording typical hippocampal activity.

Figure 2 shows two hippocampal electrodes positioned in holders of the stereotaxic instrument. Both electrodes are already inserted and firmly attached to the skull by means of dental cement (Kadon). One electrode is connected to the input panel of an EEG recorder for monitoring hippocampal activity. The two other electrodes in the foreground are already clipped off to proper length and well-embedded in dental cement.

All electrodes, including those from six cerebral cortical areas (stainless steel screws in Figure 3) for conventional EEG recordings, were soldered to a subminiature connector, and the entire assembly was attached permanently with dental cement to the skull of the animal (Figure 3). The wound around the connector was closed with deep and superficial sutures (Figure 4).

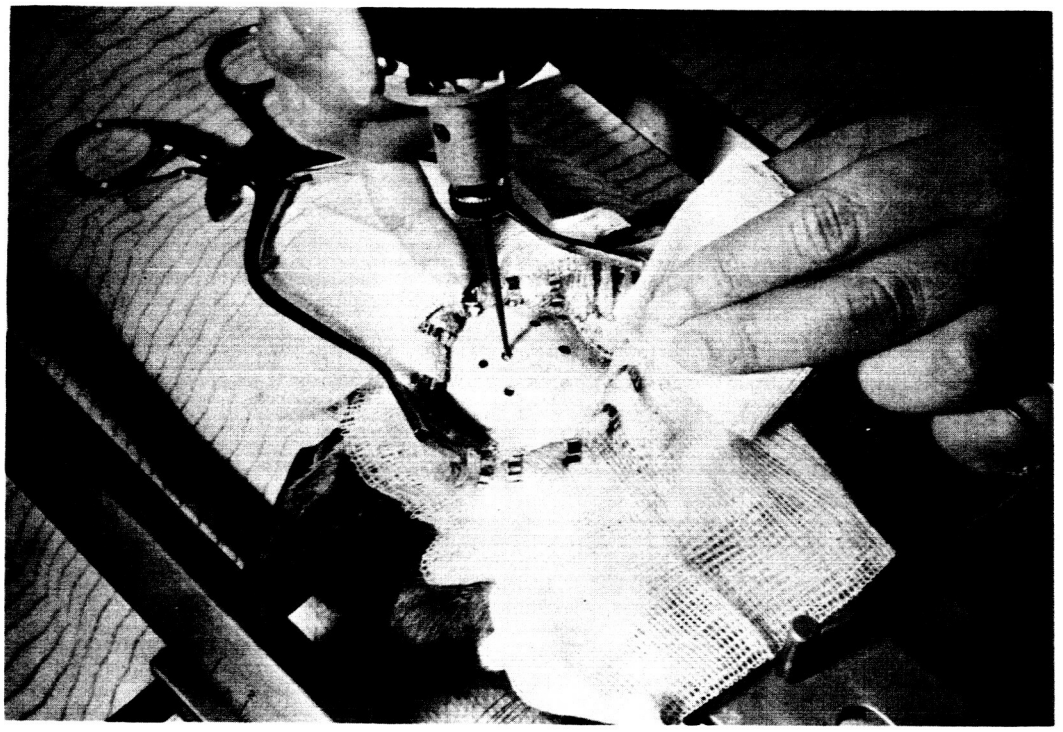

Figure 1. Operation for chronic implantation of brain electrodes. Exposure of parts of the skull and drilling holes over the site of planned implantation.

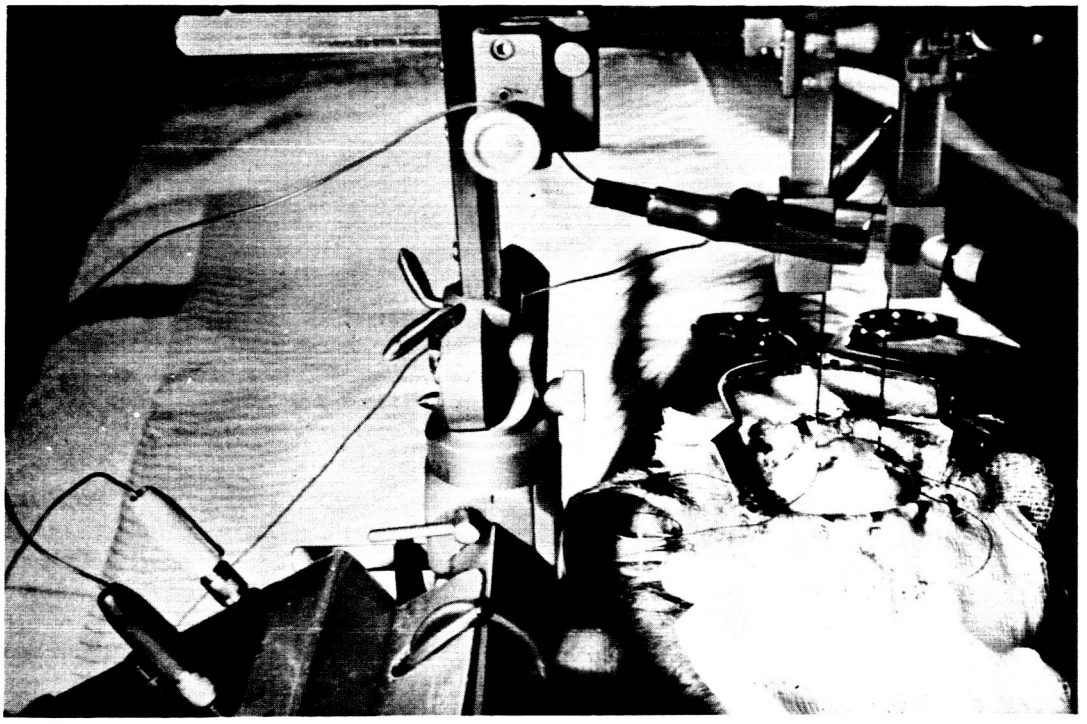

Figure 2. Implantation of bipolar electrodes with the aid of a stereotaxic instrument. For details see text.

Figure 3. Mounting of microconnector to the skull with white dental cement.

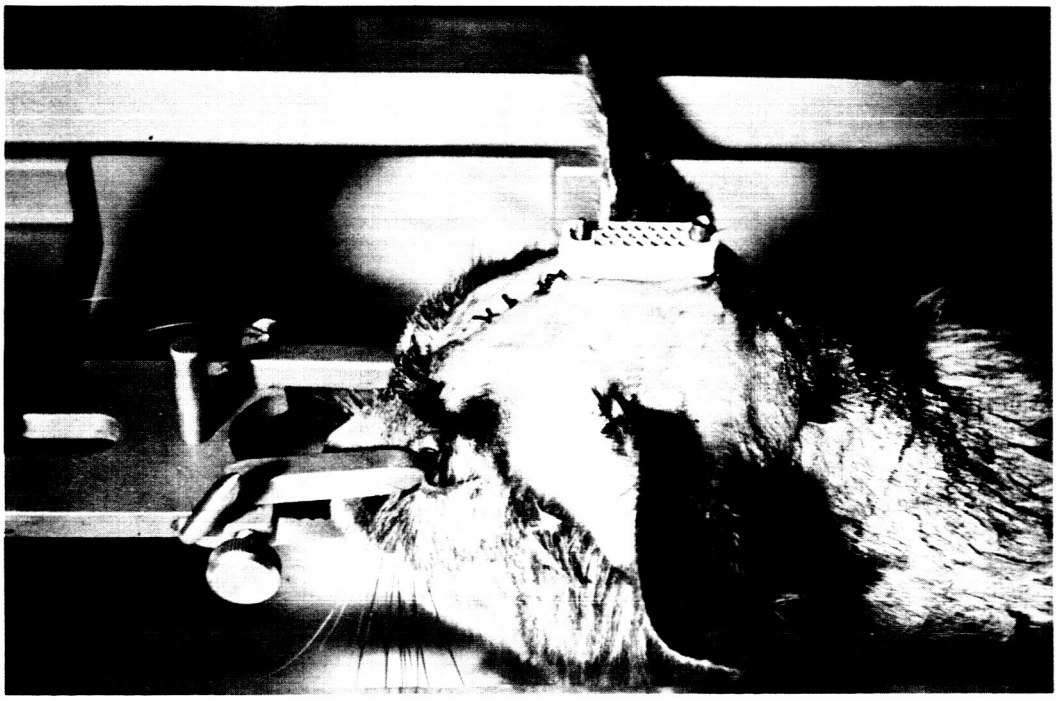

Figure 4. Cat positioned in stereotaxic instrument at the very end of the operation.

Figure 5. Typical view of an experimental cat (L-2) with 13 chronically implanted stainless steel recording electrodes. Recording cable attached. Picture taken prior to irradiation.

Special post-operative care including antibiotic coverage was given. Recuperation of the experimental animals was excellent. A typical view of a fully recovered chronic cat with an attached recording cable is demonstrated in Figure 5.

Equipment Used. Operations and recording sessions were carried out in a large (7' x 7') doubly-shielded room with a heavy (600 lbs.) vibration-free table in order to conduct experiments without electrical or mechanical disturbance from extraneous sources. EEG records were made with a Grass 8-channel EEG recorder. For electrical stimulation through implanted electrodes Grass (Model S-4) square-wave tissue stimulators with isolation units were used. Light flashes for visual stimulation were provided by a Grass (Model PS-2) photostimulator. Auditory stimulation was accomplished through a 15 watt loudspeaker mounted on top of the animal recording cage. Auditory stimuli consisted either of clicks or of signals of various frequencies and duration.

EEG data were also stored on magnetic tape by means of an FM tape recorder for further processing by a CAT computer (Computer of Average Transients). Part of the stimulating and recording system used is shown in Figure 6.

An electronic system for impedance measurements in the central nervous system was devised and assembled by the investigators. The apparatus, consisting principally of an AC generator, a Wheatstone resistance and capacitance bridge, preamplifiers, and tuned filters, made precise impedance measurements in biological tissue possible, with identification of resistance and capacitance components at frequencies from 1 KC to 100 KC, and with signal voltages in the hundred microvolt range, or less.

Figure 7 is a block diagram of this apparatus. Signals at frequencies from 1KC to 100 KC were supplied through a high frequency

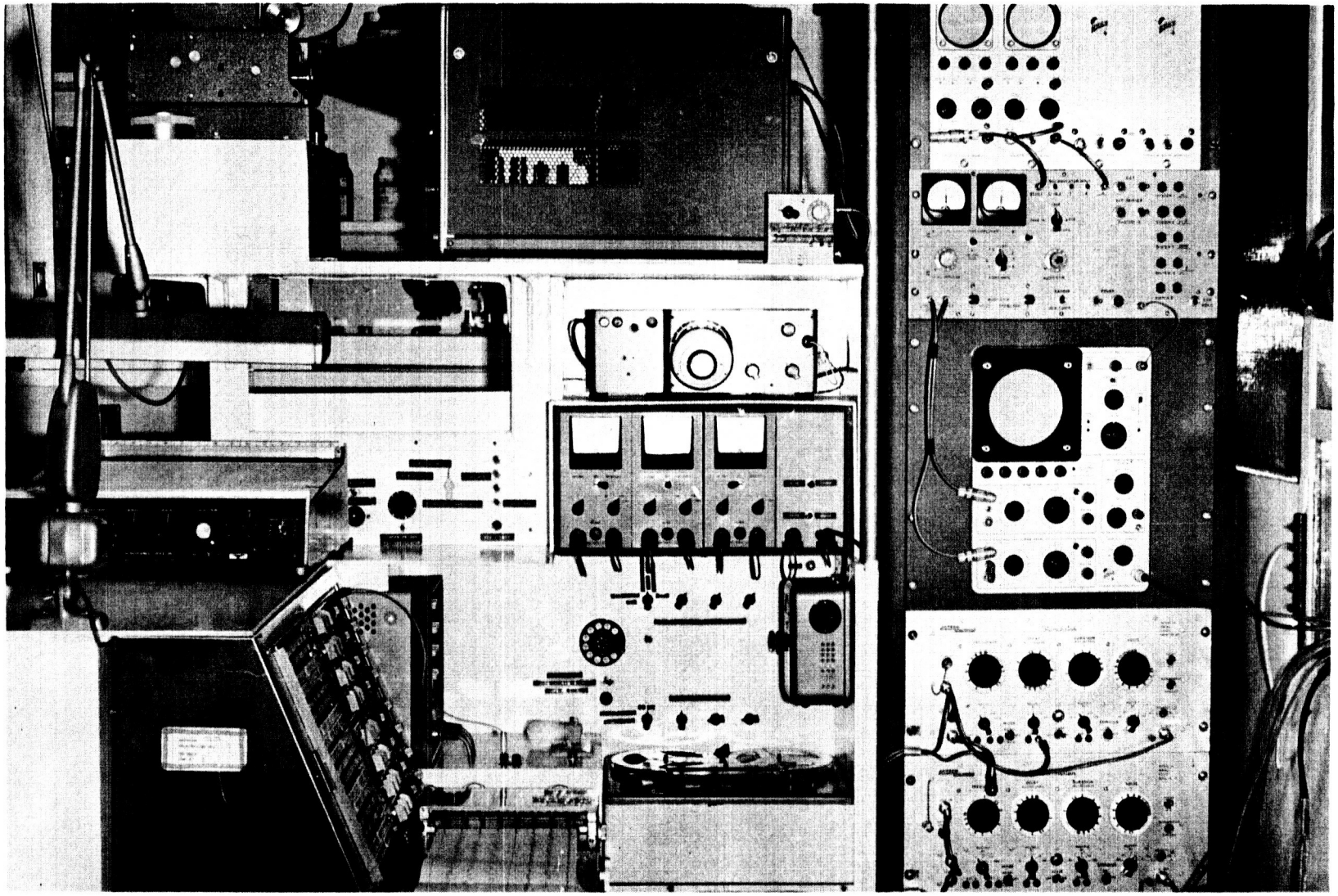

Figure 6. Neurophysiology laboratory. Part of the stimulating and recording system as used during the described experimentation.

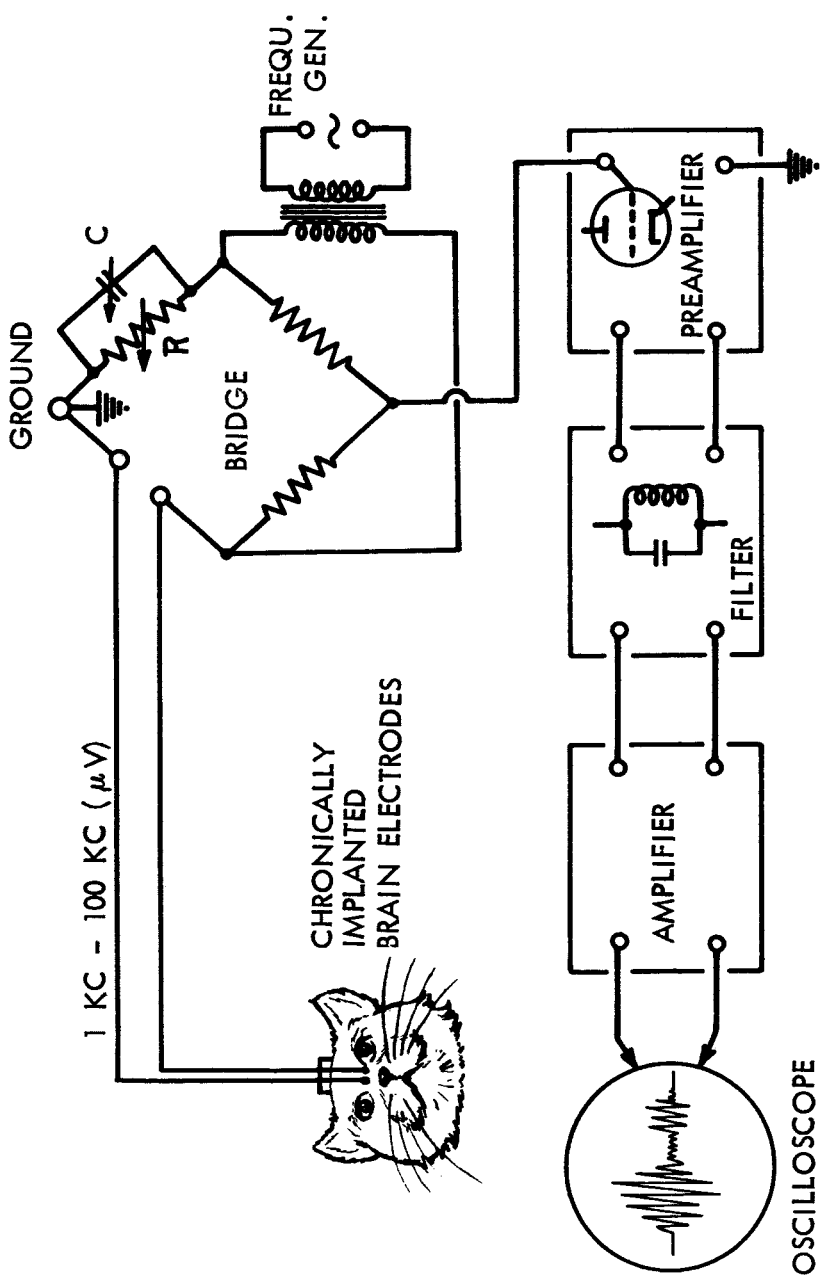

FIGURE 7. - BLOCK DIAGRAM OF THE EXPERIMENTAL SET-UP USED FOR IMPEDANCE MEASUREMENTS WITH MICROVOLT SIGNALS IN THE BRAIN THROUGH DEEPLY SITUATED AND CHRONICALLY IMPLANTED BIPOLAR ELECTRODES.

isolation coil via a Wheatstone bridge into the brain of the experimental animal. Signals obtained from the bridge were passed through a preamplifier, a variable narrow band filter, and an AC amplifier into a Tektronix type 502 A Oscilloscope. Balancing of the bridge was accomplished by coarse adjustment of a precision step-up attenuator (covering 50 to 5,000 ohms) and a capacitance selector (covering 350 μμ F to 0.027 μ F); fine adjustment of the bridge was done by means of a precision 10-turn potentiometer (200 ohms) and a variable capacitor (20 to 365 μμ F).

The values for capacitance C and resistance R were read on calibrated dials of the adjustment knobs.

Small, spontaneous transient changes in impedance were recorded by rectifying signals of the unbalanced bridge with subsequent recording on a Sanborn 550 M recorder.

For body shielding during head X-irradiation a lead-shielded cylindrical container was designed and fabricated. The unit rotated at 1 rpm, and was designed for use with horizontal beams of a 250 KV X-ray source. Figure 8 shows a cat restrained in the shielding cylinder prior to X-ray exposure.

3. Experimental Results

Pre-and post-irradiation EEG records were taken frequently. During critical periods of experimentation up to four recording sessions were held daily. Two cats were subjected to (250 KV) head X-irradiation, the first at 4,000 r, and the second at 2,000 r. Following irradiation to the head, there was observed: marked epilation in the exposed areas, impairment of vision, chronic infection of the upper respiratory tract, and diminished appetite with consequent development of a general debilitated appearance. Figure 9 shows a cat 250 days after exposure to 2,000 r head X-irradiation. The appearance of the animal at this state of experimentation is in great contrast to that prior to irradiation as shown in Figure 5.

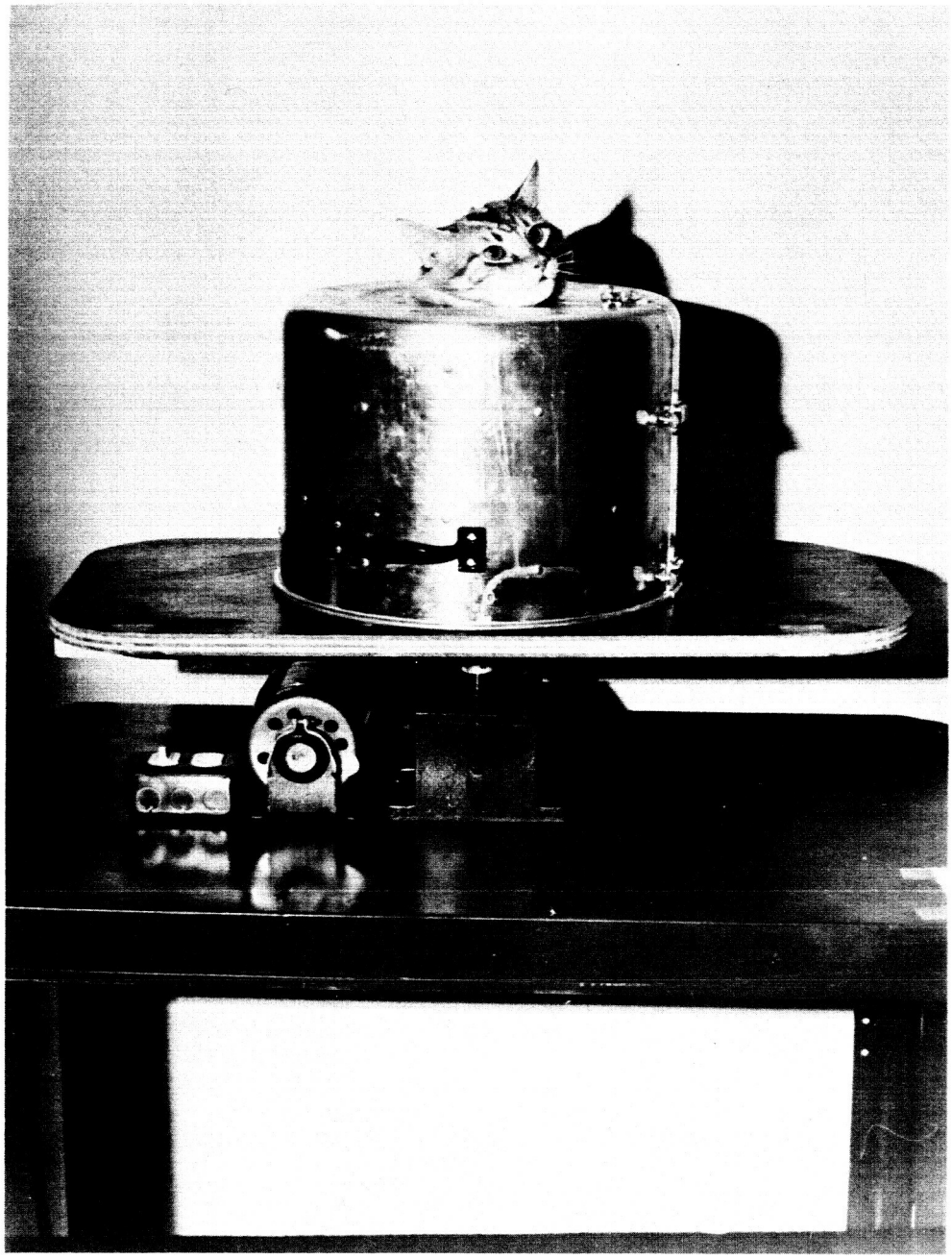

Figure 8. Chronically implanted cat (L-5) positioned in lead-shielded cylindrical container providing body shielding during head X-irradiation.

Figure 9. Experimental cat (L-2), 250 days after exposure to 2,000 r head X-irradiation. Note epilation and debilitated appearance in comparison with pre-irradiation status as shown in Figure 5.

This animal, and the other animals irradiated, displayed significant neuropathological signs such as weakness of the limbs (particularly of the hind limbs) and depressed reflex activity. In addition, the cat exposed to 4,000 r head irradiation showed a pronounced drop in exploratory activity and a progressive drowsiness which ended in a stuporous state.

EEG Recordings:

Bioelectric changes in cortical and subcortical areas following head X-irradiation are demonstrated in the following seven charts (Figures 10 - 16): Figures 10, 11, and 12 summarize our observations for a cat exposed to 4,000 r head X-irradiation. Changes of cortical EEG (Figure 10) consisted of a gradual slowing of electrical activity with an increase in amplitude. This effect was fully apparent as early as 53 hours after exposure. Figure 10 also shows the susceptibility of the dorsal hippocampus to irradiation. Two to three days after exposure the pattern of hippocampal activity was practically isoelectric. The effect of 4,000 r head irradiation on subcortical areas is demonstrated in more detail in Figure 11: Theta-synchronization of the hippocampus as shown in the pre-irradiation control was gradually diminished until about two to four days after head irradiation the hippocampal lead became almost isoelectric. Normally fast amygdaloid activity gradually changed its character by showing a considerable slowing of activity and increase in amplitude. Finally (at 10 days; one day before death) the overall activity of the basal amygdala was very depressed.

Four thousand r X-irradiation to the head also had a significant effect on the cortical and subcortical arousal pattern of the animal. Data concerning this effect are summarized in Figure 12. Bars below the record indicate the duration of a continuous intense tone of 5,000 cps to which the animal was exposed. There was slight enhancement of cortical and subcortical arousal up to 19 hours post irradiation.

EEG AND HIPPOCAMPAL CHANGES FOLLOWING 4000R HEAD IRRADIATION (CAT LI)

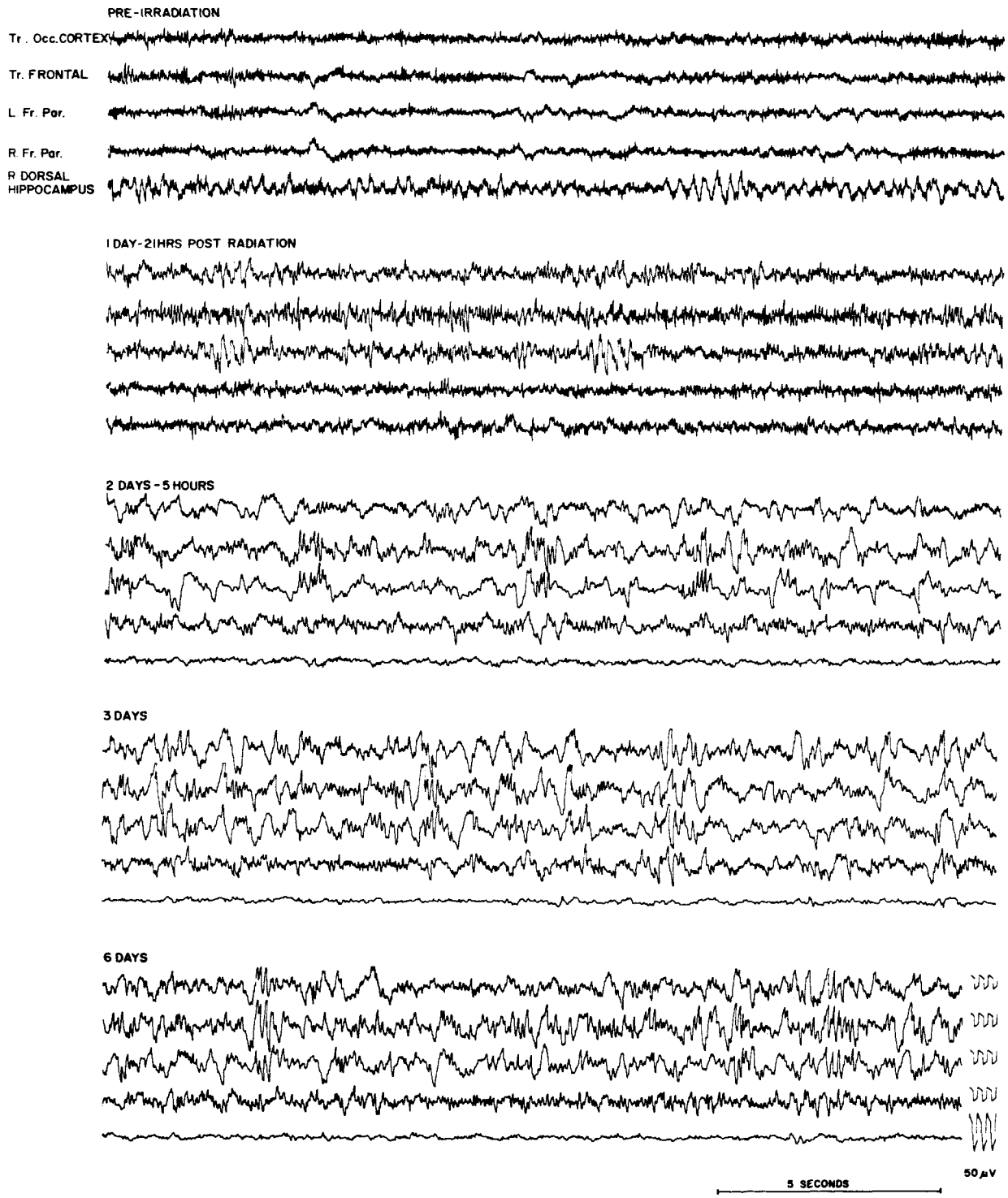

Figure 10. EEG and hippocampal changes following 4,000 r head X-irradiation. Cat L-1. For details see text.

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CHANGES IN HIPPOCAMPAL AND AMYGDALOID ELECTRICAL ACTIVITY FOLLOWING 4000R, CAT LI

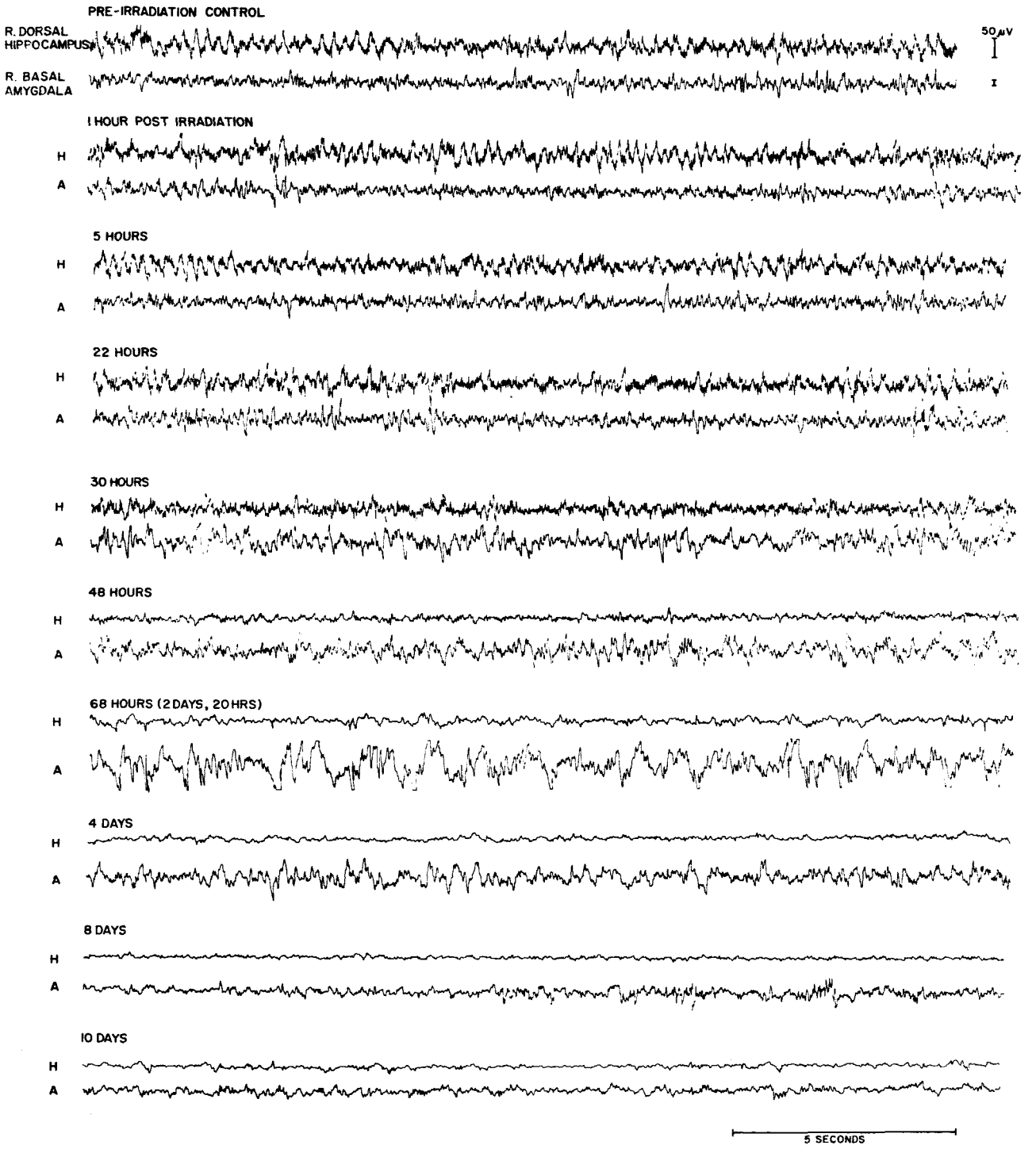

Figure 11. Changes in hippocampal and amygdaloid electrical activity following 4,000 r head X-irradiation. Cat L-1 For details see text.

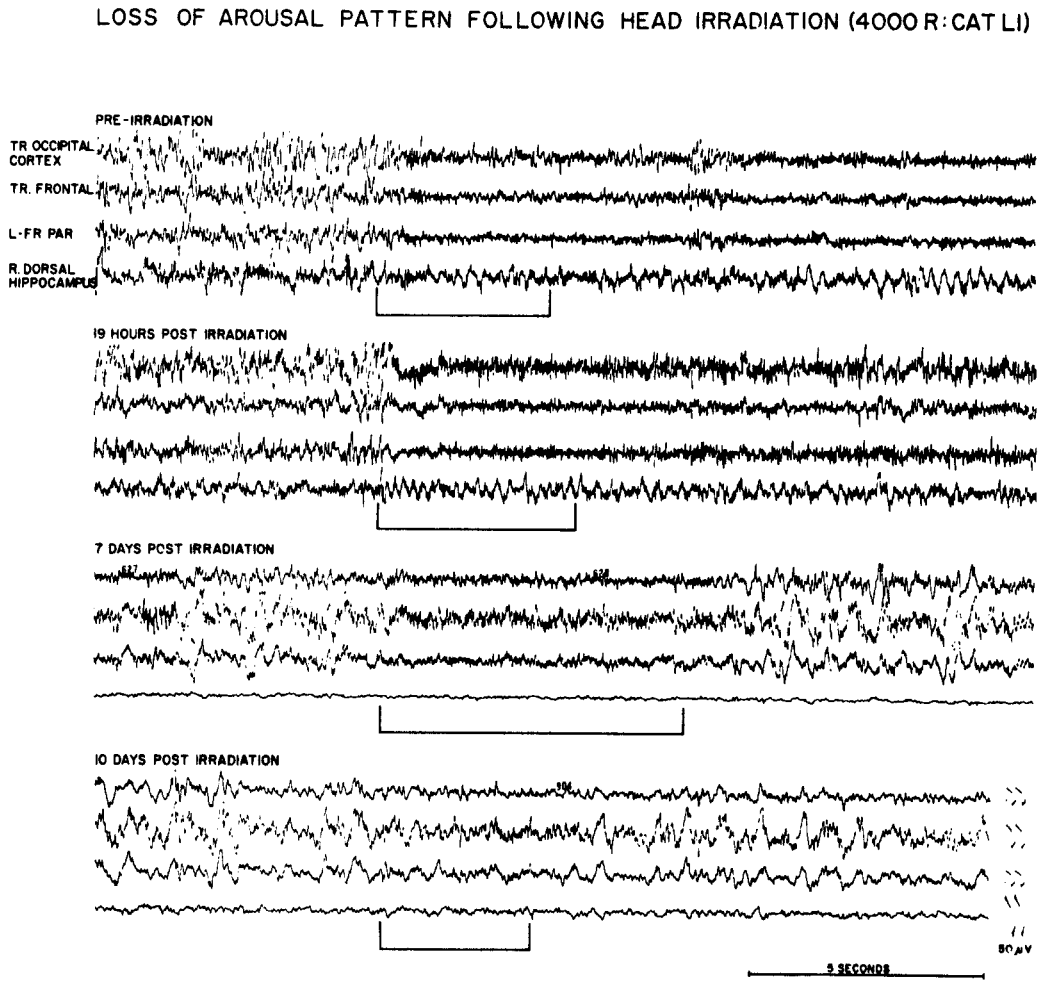

Figure 12. Loss of arousal pattern following 4,000 r head X-irradiation. Cat L-1. For details compare text.

Seven days after irradiation a certain degree of cortical arousal was possible only during duration of intense auditory stimulation. There was, however, no hippocampal response. Finally, (at 10 days; one day before death) the animal was completely stuporous and could not be aroused at all.

Another cat, which was exposed to 2,000 r head X-irradiation, survived for more than 300 days. Changes of cortical and subcortical activity as a function of time after irradiation are summarized in Figures 13 through 16. Figure 13 stresses the early development of bioelectrical abnormality in the central nervous system, particularly in subcortical areas. As early as 18 hours post irradiation the right and left amygdaloid leads began to show distinct spontaneous spikes in the range of 75 to 80 μ V amplitude. Finally, at 7 days after irradiation, there were unquestioned specific spike and wave complexes in the left amygdaloid lead. Up to 4 days following head irradiation there seemed to be an increased cortical electrical activity which then was followed, after about a week, by a slowing of activity. In contrast to all pre-irradiation recordings where the aroused animal showed a very active cortex, a theta-synchronization in the hippocampus, and fast amygdaloid activity, the cortical pattern seven days after X-ray exposure began to show a 3 to 5 cps rhythm that has been seen to accompany head irradiation (also previously shown in Figure 10).

Later recordings of cortical activity are marked by the development of cortical spike-wave patterns. Figure 14 demonstrates the appearance of these spikes and stresses the correlation of these cortical effects with bioelectric abnormalities as they appear in subcortical areas. Spontaneous definitive spiking waves were not observed in the frontal cortex before the 98th day post irradiation. After more than 300 days this spiking activity was even more pronounced. A comparison of cortical and subcortical phenomena, as seen in this chart and also in Figure 13, stresses the fact that cortical

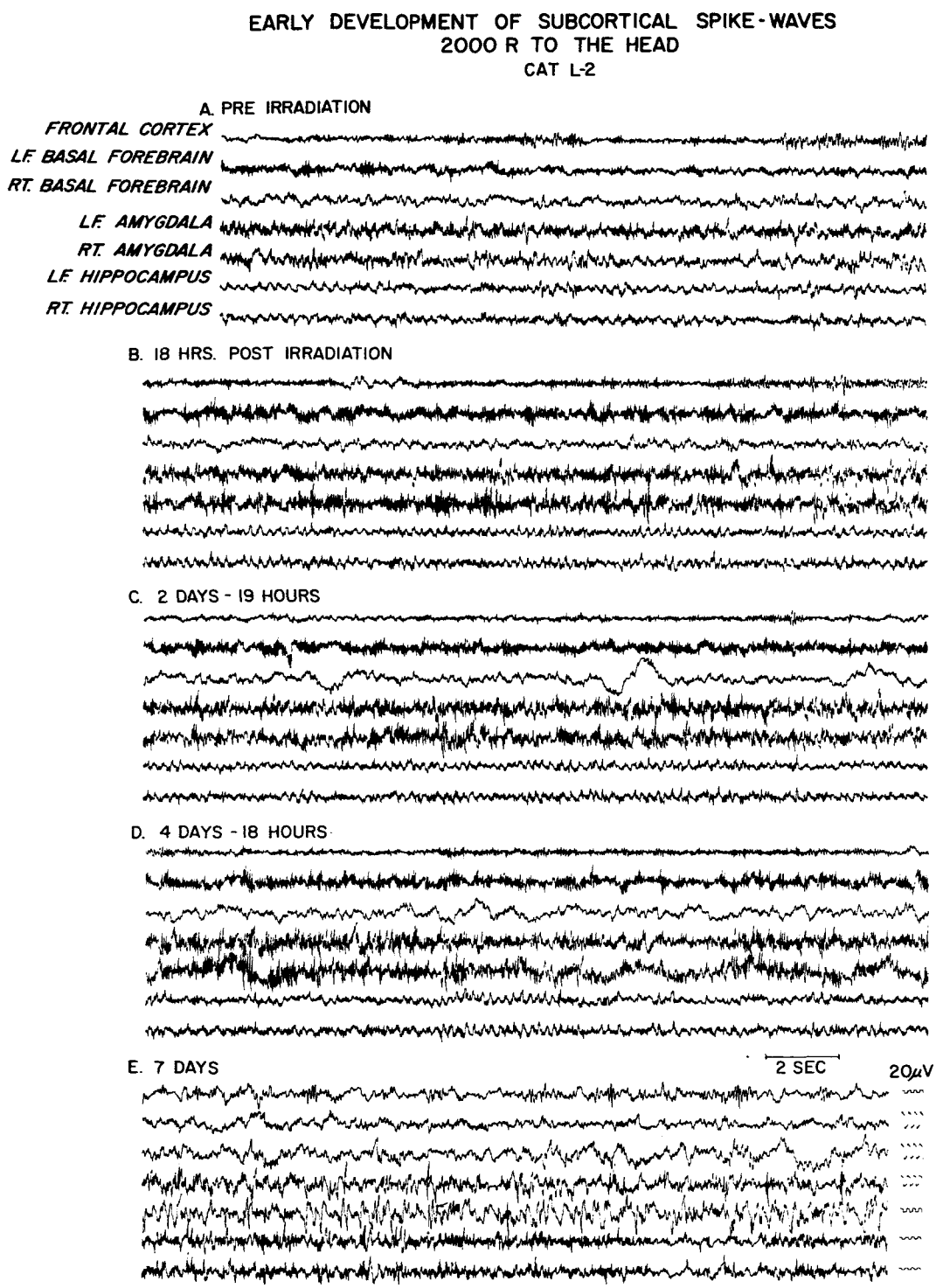

Figure 13. Early development of subcortical spike-waves following 2,000 r head X-irradiation. Cat L-2. For details see text.

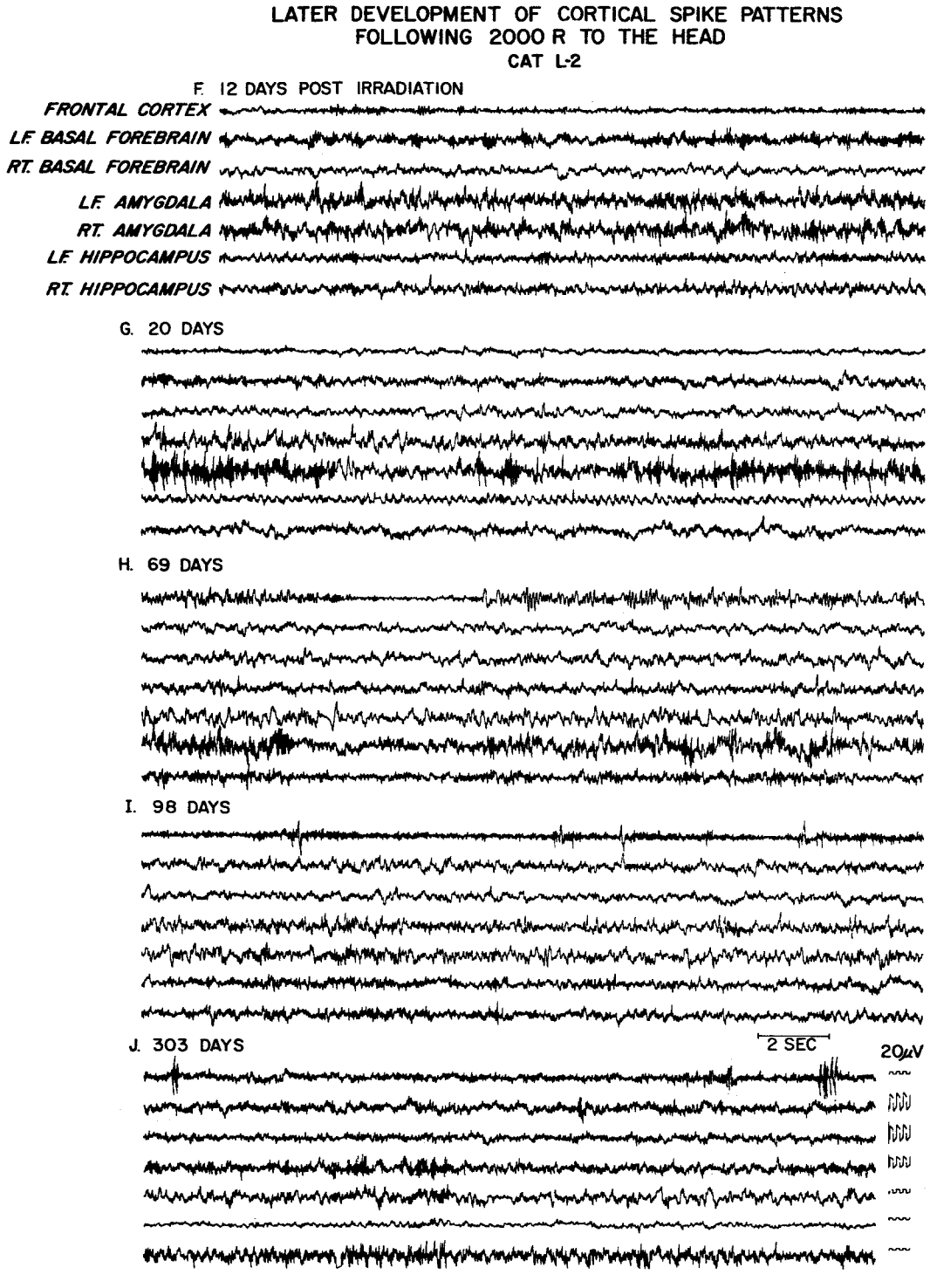

Figure 14. Later development of cortical spike patterns following 2,000 r head X-irradiation. Cat L-2. For details see text.

post-irradiation effects appear later than subcortical effects. Of all subcortical areas from which we recorded, the amygdaloid nuclei seemed to be amongst the most critical sites.

Another important finding in a cat exposed to 2,000 r head X-irradiation is shown in Figure 15. This chart stresses the development of spontaneous cortical and subcortical seizures gradually involving more and more of the nervous system. For example: 154 days after head irradiation seizure patterns not only were present in the cortical areas but also became apparent in subcortical areas such as the caudate and amygdaloid nuclei. It is important to notice that the short discharge bursts occur approximately every 9 to 16 seconds. A comparison of discharge amplitudes 203 days after irradiation with those of earlier records indicates that the amplitudes of the discharge patterns seem to increase and to intensify with time. In fact, it was shown that intensification of these discharge bursts could be carried to a point where the entire nervous system was involved in a grand mal seizure. This reaction is demonstrated in Figure 16. The bars on top of the records indicate the duration of a continuous intense tone of 5,000 cps, to which the animal was exposed. Recording commenced a few seconds after intravenous injection of 2.6 mg/kg Metrazol (total dose 6 mg). It can be seen from the continuous records that intense auditory stimulation caused increasing discharge activity in the frontal cortex and - if continued for sufficient time- finally led to the development of a typical grand mal seizure. A control experiment in a chronically implanted but un-irradiated cat demonstrated that the same amount of the drug Metrazol per kg together with long and intense auditory stimulation was not capable of inducing discharges and seizures. This is an indication that the functions of the brain in our head X-irradiated animal were altered to such an extent that application of Metrazol plus auditory stimulation was capable of inducing a grand mal seizure.

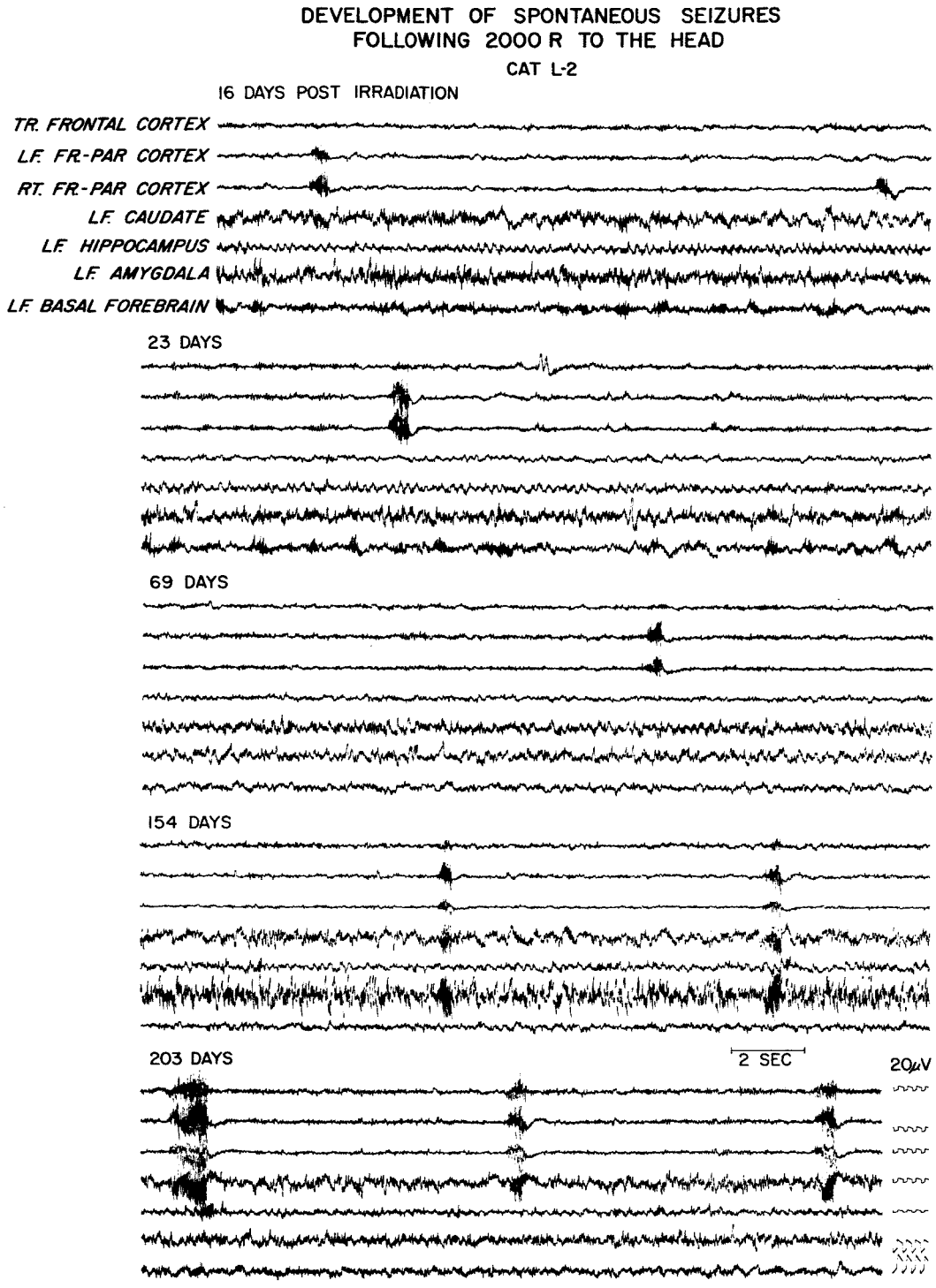

Figure 15. Development of spontaneous seizures following 2,000 r head X-irradiation. Cat L-2. For details see text.

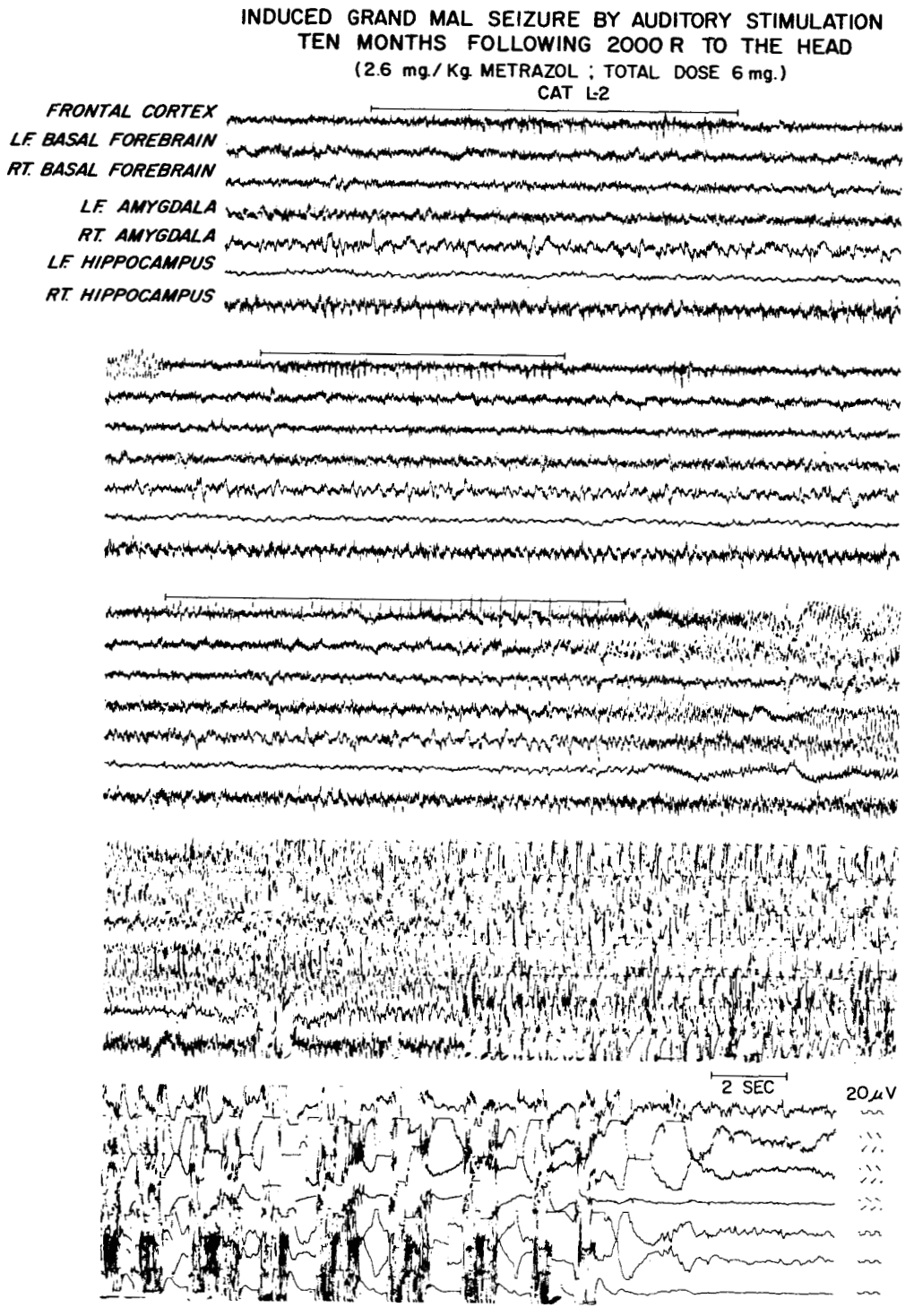

Figure 16. Induced grand mal seizure by auditory stimulation 10 months following 2,000 r head X-irradiation. Cat L-2. For details see text.

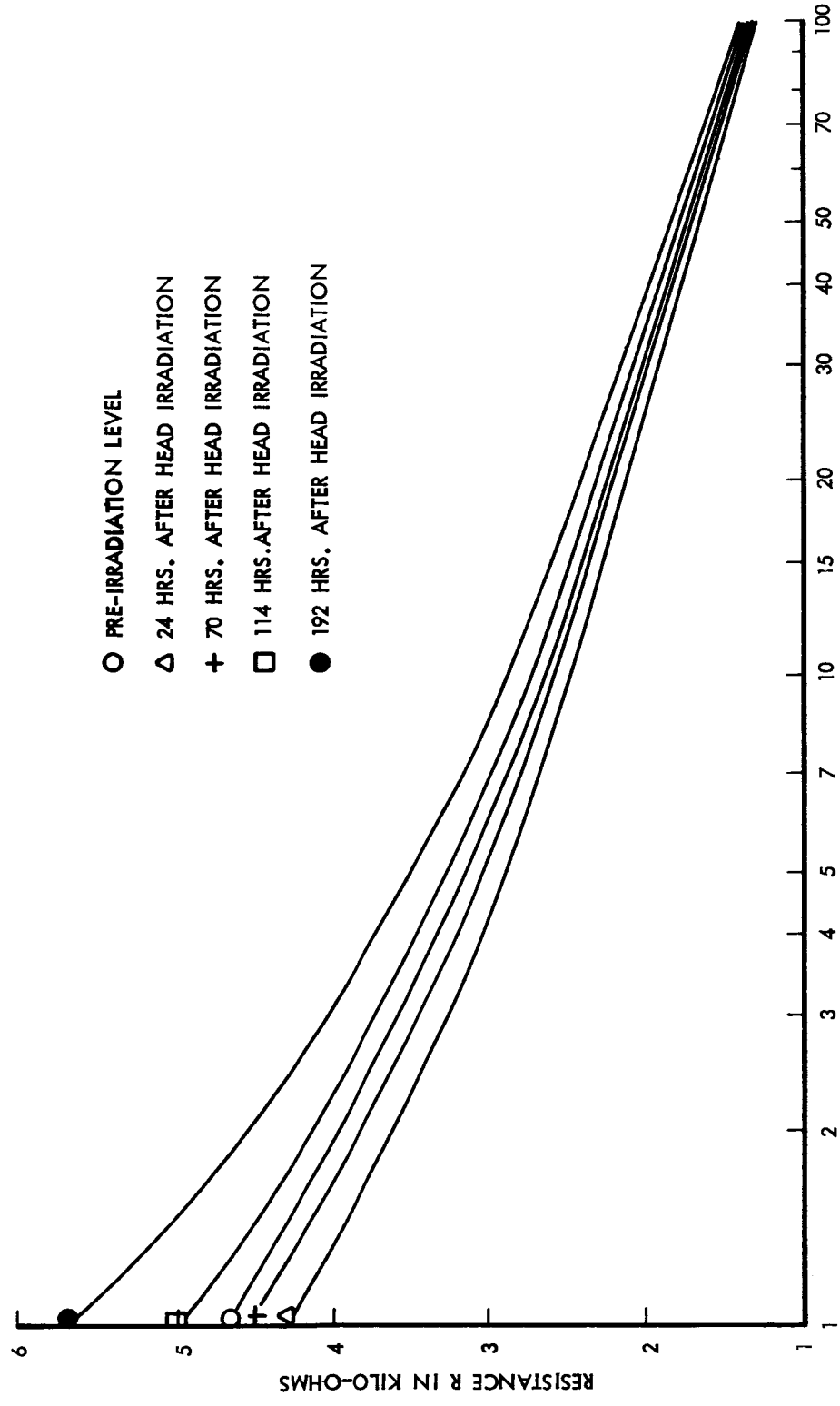

FIGURE 17. - IMPEDANCE CHANGES IN THE LEFT HIPPOCAMPUS OF A CHRONICALLY IMPLANTED CAT (L-2) FOLLOWING 2,000 r (250 KV) HEAD X-IRRADIATION. RESISTANCE R IN KILO-OHMS IS PLOTTED VERSUS SIGNAL FREQUENCY IN KCPS.

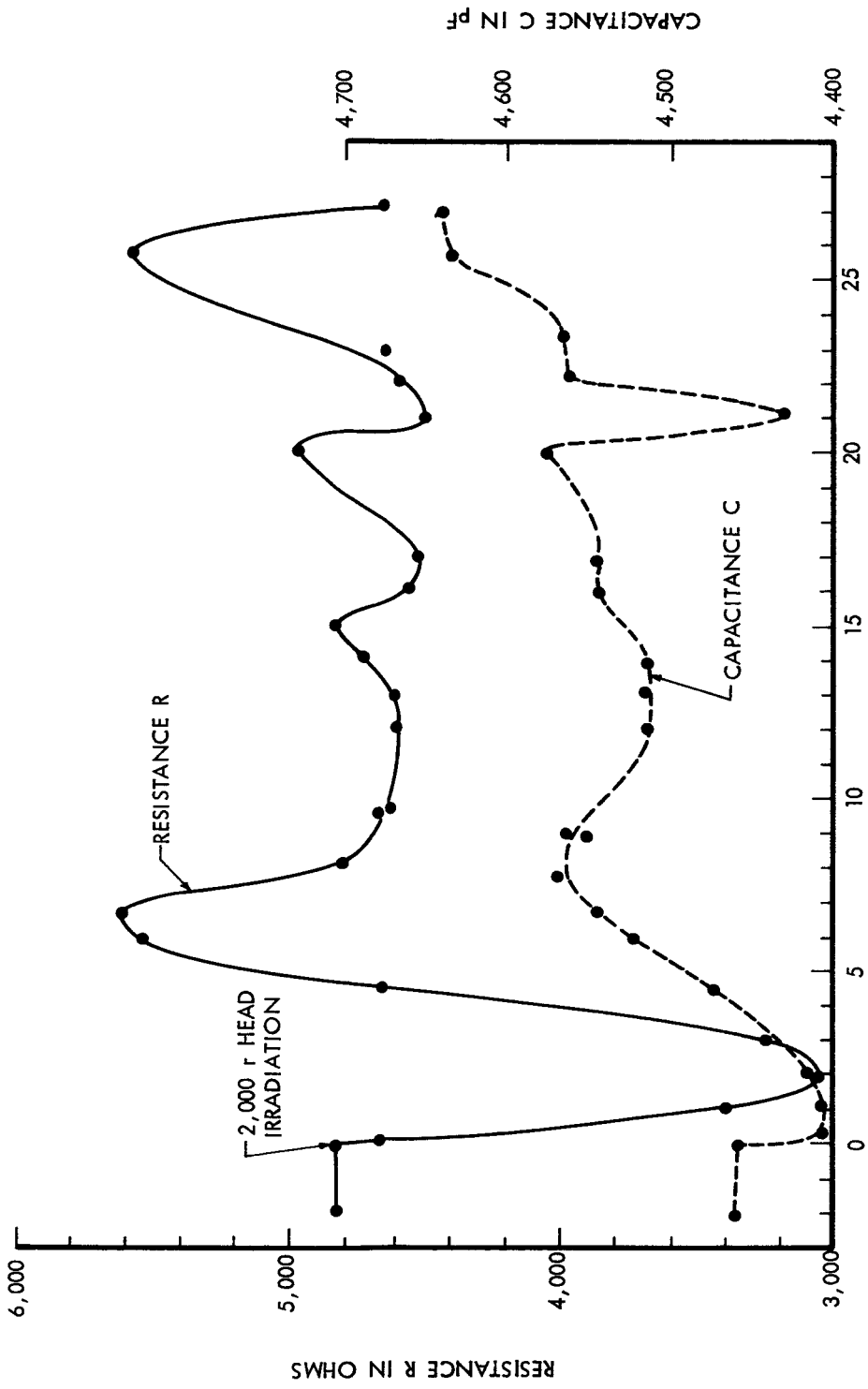

FIGURE 18. - IMPEDANCE EXCURSIONS IN THE LEFT HIPPOCAMPUS OF A CHRONICALLY IMPLATED CAT (L-2) FOLLOWING 2,000 r (250 KV) HEAD X-IRRADIATION. CHANGES OF RESISTANCE R AND CAPACITANCE C WERE FOLLOWED OVER A PERIOD OF 27 DAYS AFTER IRRADIATION. MEASUREMENTS AT 5 KCPS.

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Impedance Measurements:

Preliminary impedance measurements in the hippocampus were carried out prior to and after 2,000 r head X-irradiation. Figure 17 shows the measured resistance R of a pool of neurons and neuroglial cells between the poles of a bipolar strut electrode positioned in the left hippocampus, plotted versus the applied signal frequency in kilocycles per second. Measurements were made before and up to 230 hours after X-ray exposure. It can be seen from Figure 17 that following irradiation the resistance changes Δ R were comparatively large at lower frequencies, whereas at higher signal frequencies, due to capacitive coupling, resistance changes became smaller and smaller. The nature of Δ R, however, i. e. positive or negative deviation, remained constant throughout all signal frequencies. Following irradiation there was a decrease in resistance with subsequent rise after 70 to 80 hours. This effect is better shown in Figure 18, where resistance and capacitance changes in the left hippocampus are shown as a function of elapsed time for 27 days following X-ray exposure. Immediately after irradiation both resistance and capacitance began to show large excursions. Maximum excursions in resistance and capacitance do not seem to coincide precisely in time, although there is some correlation in the two graphs. A cautious interpretation of these preliminary data is attempted in the discussion.

Histopathological Findings:

Autopsies were carried out upon completed experimentation. A large number of histological serial sections of irradiated cat brain were studied and the effects of ionizing irradiation determined histopathologically. Figures 19 through 23 are examples of some acute vascular and perivascular reactions in various areas of a cat brain 10 days after 4,000 r (250 KV) head X-irradiation. Intergyral vasculitis (Figure 19), intracerebral vasculitis (Figure 20), and perivascular neutrophilic infiltration (Figure 21) could be readily detected.

Figure 22 demonstrates extravascular leucocytic infiltration in the cerebral cortex. Degenerative changes in the hippocampus such as the appearance of "ghostcells", lysis, and general dissolution are shown in Figure 23.

Histological examination also provided information about the exact location of the tips of chronically implanted electrodes.

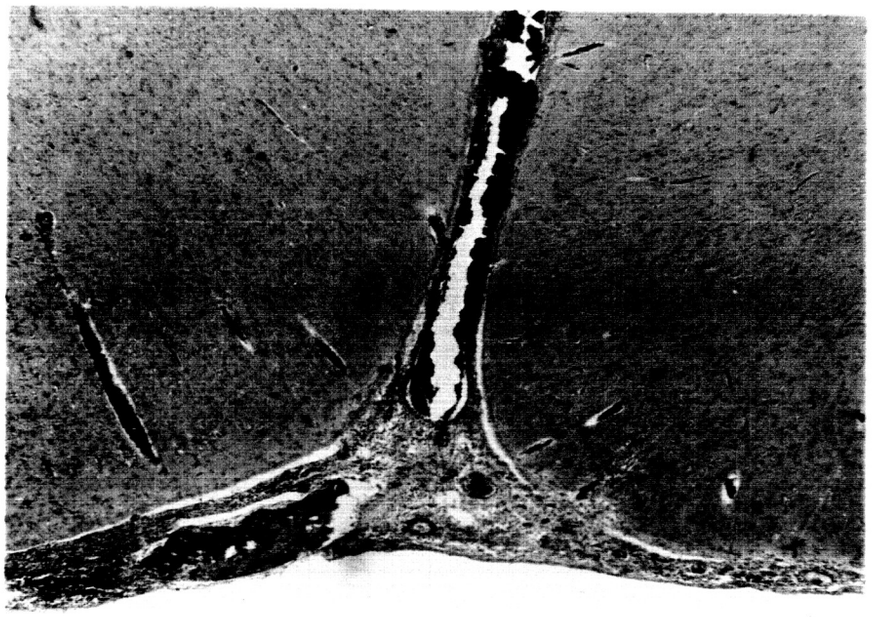

Figure 19. Intergyral vasculitis in cat brain 10 days after exposure to 4,000 r head X-irradiation. X 36.

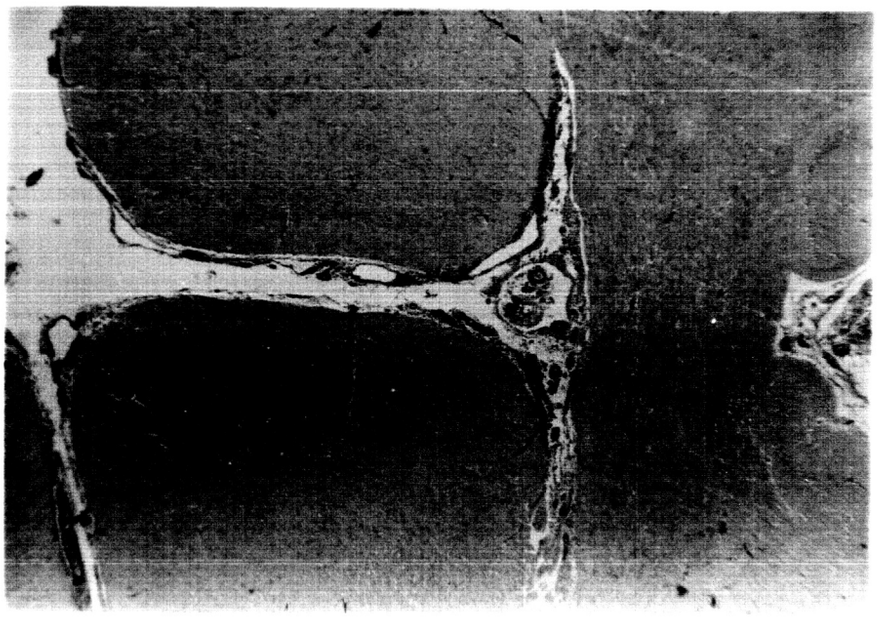

Figure 20. Intracerebral vasculitis in cat brain 10 days after exposure to 4,000 r head X-irradiation. X 36.

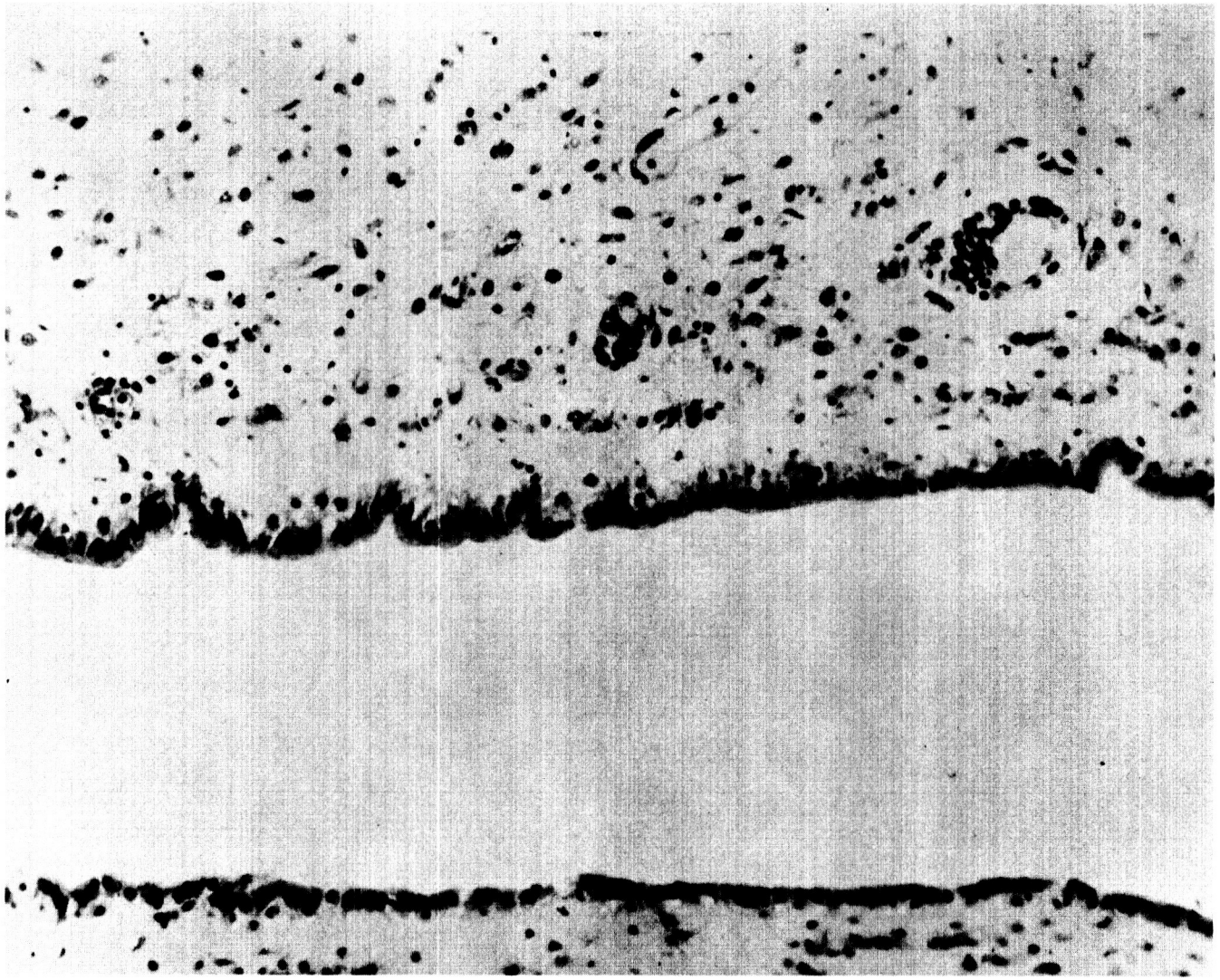

Figure 21. Radiation damage in cat brain (L-1) 10 days after exposure to 4,000 r (250 KV) head X-irradiation. Perivascular neutrophilic infiltration in the hypothalamus (3rd ventricle adjacent). X 600.

Figure 22. Histopathological changes in cat brain (L-1) 10 days following 4,000 r head X-irradiation. Extravascular leucocytic infiltration in the cerebral cortex. X 400.

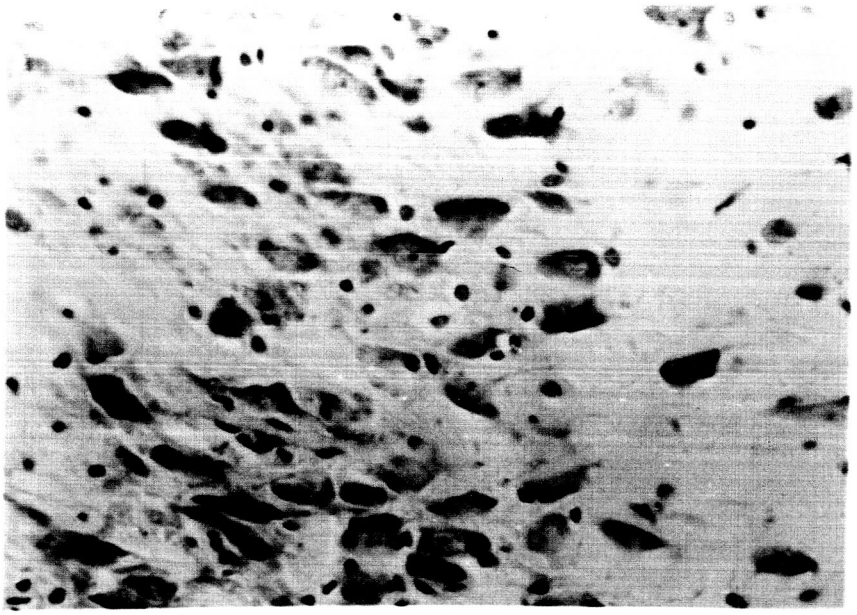

Figure 23. Histopathological changes in cat brain (L-1) 10 days following 4,000 r head X-irradiation. Degenerative changes in the hippocampus; ghostcells, lysis, and dissolution. X 800.

III. THE ELECTRICAL CHARACTERISTICS ASSOCIATED WITH NEUROPHYSIOLOGICAL PROCESSES OF NEURONS AND NEUROGLIA IN TISSUE CULTURE AND THE SUBSEQUENT EFFECTS OF IONIZING RADIATION.

1. Pertinent Literature

Although a certain amount of information exists on the effects of ionizing radiation on the nervous system in animals, one of the unresolved problems is whether the results are due to direct effects of irradiation on the nerve cells or are indirect effects due to ionizing damage of glial elements or of the brain vasculature (Haley and Snider (21)).

An ideal method to separate brain cell constitutents from each other and to make them individually available for experimentation is the tissue culture method. Groundwork was laid by Pomerat (33) who compiled a large body of knowledge concerning the identity and morphological characteristics of surviving neurons and neuroglial cells in tissue culture. From cultures of chick embryo spinal ganglion, Crain (7) demonstrated that nerve cells can retain morphological as well as functional bioelectric capabilities in vitro. Hild, Chang, and Tasaki (22) described for the first time the singular and interesting electrical properties of astrocytic glia in tissue culture, and the question of whether or not the glial cells in vivo have the same properties was discussed by Tasaki and Chang (41). Wardell (44) recently confirmed the existence of typical glial responses to extracellular stimulation but pointed out that similar "responses" could be obtained from mesodermal cells in the same culture. Hild and Tasaki (23) studied the morphological and electrophysiological responses of neurons and neuroglial cells in tissue culture. They were able to obtain both extracellular and intracellular spontaneous and evoked neuronal action potentials and neuroglial responses. More complex bioelectric activity evoked by electrical stimuli was recorded by Crain and Peterson (8) who in their experiments with cultured spinal cord demonstrated a re-

markable degree of functional neuronal organization in vitro.

Recent theories of neuronal function appear to implicate the neuroglia directly with the physiology of neuronal discharge (Hyden and Pigon (26); Galambos (12)). That the neuroglia may be important in regulating the passage from the blood to the brain, and of neuronal metabolites from the brain into the blood, has also been postulated (Tschirgi (42)).

To our knowledge, no one has reported any research findings combining electrophysiological studies of neurons and neuroglia in tissue culture and the effects of ionizing radiation on the functional properties of cultured cells.

2. Methods and Materials

Tissue Culture Techniques:

The techniques used for preparing and maintaining explants from the central nervous system in tissue culture were principally those described by Hild and Tasaki (23). In all cases three day old albino rats[1] (Sprague-Dawley) were sacrificed for explants. The procedures for dissecting the newborn rats, removing the brain tissue, and subsequent culture of the explant were carried out under aseptic conditions.

Cultivation was accomplished by affixing a 2 mm^2 explant to a No. 1 coverslip 8 x 50 mm by means of a chicken plasma[2] clot. The coverslip containing the explant was inserted into a roller tube 15 x 150 mm and 1.5 ml of medium added aseptically. The roller tube containing the explant was placed in a water-jacketed incubator at 37°C. The cultures were rotated in a roller drum at 12 revolutions per hour (see Figure 24). The medium consisting of 50% Gey's Balanced Salt Solution (BSS), 45% inactivated calf serum[3], 5% embryo extract[3] (from eight day-old chick embryos), and 100 units per ml of penicillin G[4]

1. Berkeley Pacific Laboratories, Berkeley, California
2. Difco Laboratories, Detroit, Michigan
3. Microbiological Associates, Albany, California
4. E. R. Squibb & Sons, New York, New York

was changed twice weekly. Hild and Tasaki (23) reported that 300 mg% glucose in the medium enhanced neuronal survival, glial viability, and myelin formation around axons. Their methods did not take into account glucose in the serum and embryo extract components of their medium. An enzyme assay called Glucostat[5] was used to determine glucose concentration which was subsequently raised to 300 mg%.

Preliminary studies were conducted to determine whether or not explants could be kept in culture for extended periods of time without loss of morphological or physiological integrity. Long term viable cultures would permit the study of chronic effects of such experimental parameters as radiation on the electrophysiology of neurons and neuroglia in vitro. Since it was established that 300 mg% glucose was beneficial to neuronal survival, a study was conducted to determine whether or not a higher concentration would increase the survival time of nerve cells in culture. The following concentrations of glucose were prepared in 100 ml aliquots of medium:

300 mg% (control)
600 mg%
900 mg%
1200 mg%
1500 mg%
2000 mg%
3000 mg%

Seven "flying coverslip" cultures of dorsal root ganglia from 3 day old rats were prepared for each glucose concentration. A culture was selected weekly for each concentration, observed under the phase-contrast microscope for morphological degeneration, and used for recording electrophysiological parameters. During this study the pH of the medium was 7.2-7.4.

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5. Worthington Biochemical Corp., Freehold, New Jersey

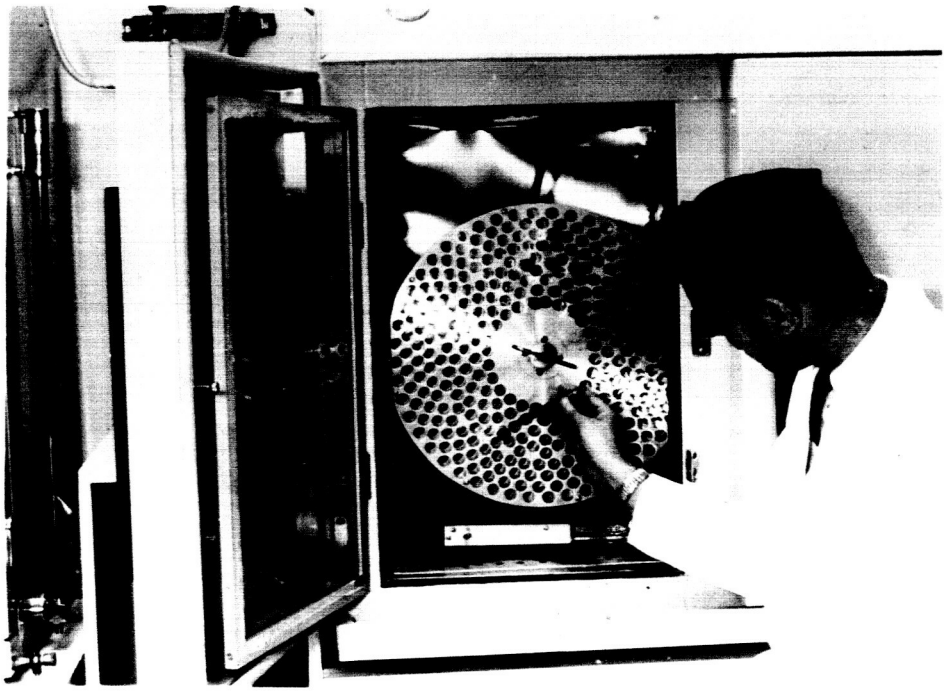

Figure 24. A Wedco roller drum containing roller tubes with cultures from the central nervous system.

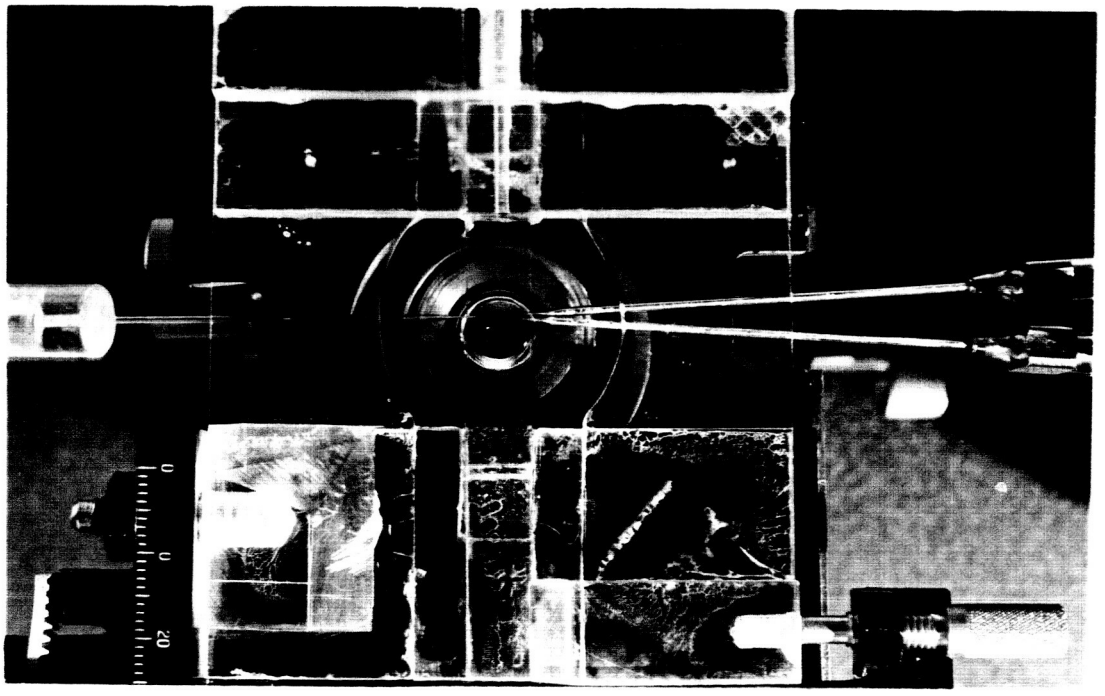

Figure 25. Plexiglass tissue culture chamber mounted on modified stage of phase-contrast microscope. Recording electrode (left) and stimulating electrodes (right) introduced.

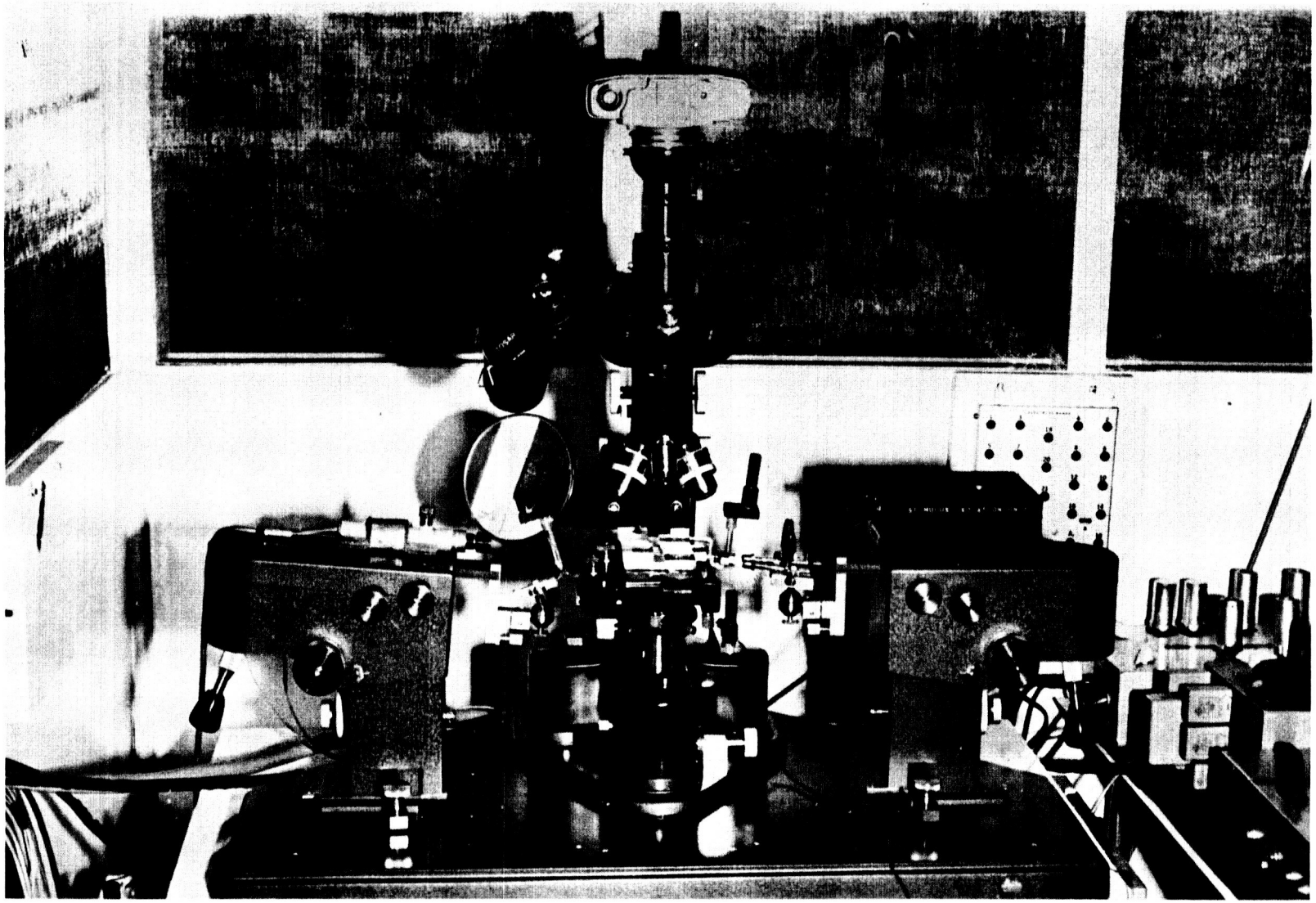

Figure 26. Leitz micromanipulator set-up mounted on heavy vibration-free table in doubly-shielded recording room. Recording and stimulating electrodes are handled with the left and right micromanipulator, respectively. The chamber containing the tissue culture explant is positioned on the stage of the phase-contrast microscope in the center.

An additional nutritional study was conducted to determine whether selective amino acid components of the medium were exhaustively utilized by the explant in culture. If this was the case, then supplemental concentrations of those amino acids might enhance the long term survival of nerve cells in vitro. Amino acid analysis was done on a Beckman Model 120 B amino acid analyzer. Samples consisted of: (1) three day-old cultures of cerebellum, (2) medium incubated for 3 days without explant, and (3) fresh unused medium as control. The protein was removed from each 20 ml sample by precipitation with 20 ml of 5% trichloroacetic acid followed by centrifugation at 25,000 rpm for 60 minutes. The supernatant of each sample was then sulfonated with 0.5M sodium sulfite for 5-6 hours at pH 7.5. The samples were dialyzed against triple distilled water for 24 hours then evaporated at 50°C to a volume of 5 ml. The procedures used for the analyses were the same as those recommended by Spackman, Stein, and Moore (39). In each case a 3 ml sample was analyzed.

Mechanical and Optical Arrangements:

The mechanical and optical arrangements used differed only slightly from those described by Hild and Tasaki (23). Two Leitz micromanipulators together with a Leitz phase-contrast microscope were mounted on a heavy (600 lbs) vibration-free table in a large doubly-shielded recording room. An experimental plexiglass chamber (as described in detail under "Technological Accomplishments") was placed on the modified stage of the phase-contrast microscope. The coverslips bearing the cultures were removed from the roller tubes and mounted in the chamber in such a way that the coverslip formed the roof of the chamber (Figure 27). The chamber was then filled through a filling and grounding tube with Gey's solution held by capillary forces (Figure 27). With the aid of the two micromanipulators, one hyperfine glass recording microelectrode and a pair of stimulating microelectrodes were introduced from below into

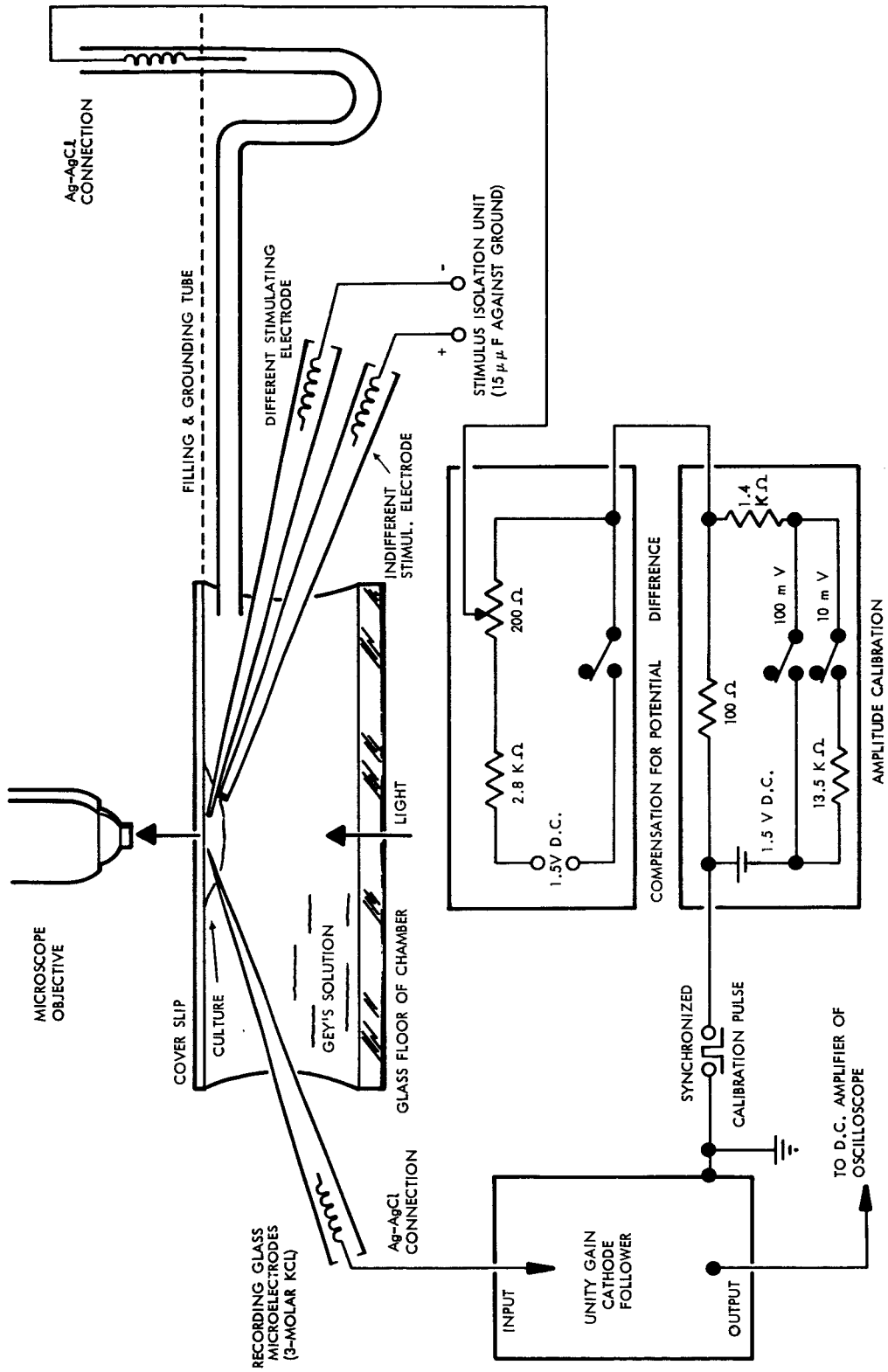

FIGURE 27. - DIAGRAM ILLUSTRATING EXPERIMENTAL ARRANGEMENT

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the open sides of the experimental chamber at angles up to 30°. Under visual inspection the microelectrodes were precisely positioned for intra and extracellular recordings. Figure 25 is a close-up photograph demonstrating the plexiglass recording chamber with the hyperfine glass recording microelectrode (left) and the two metal stimulating microelectrodes (right) in position. Figure 26 gives an overall view of the described micromanipulator set-up.

Electrical Experimental Arrangements:

Hyperfine glass micropipettes with tip diameters of less than 0.5 μ were used for intracellular recordings. The electrodes were filled with 3-molar KCL solution by the vacuum method. The resistances of these electrodes were between 35 and 45 Megohms. For extracellular stimulation a bipolar arrangement of metal-filled glass micropipettes with platinum black tips was used. Actual stimulation of excitable structures took place in the vicinity of the tip of the smaller or "different" microelectrode which was made the cathode. The metal microelectrodes which were used are described in detail in the "Appendix" of the report. A diagram illustrating the experimental arrangement is shown in Figure 27: A large Ag-AgCl electrode was used to ground the Gey's solution surrounding the culture via a U-shaped filling and grounding glass tube. Between this grounding electrode and the actual ground there were three units:

1. A small D. C. source and an attenuator for compensation of the small potential difference between the recording electrode and the grounding electrode.
2. A small D. C. source, voltage dividers, and two pushbuttons to provide calibration pulses of 10 mV and 100 mV amplitudes.
3. A synchronized stimulator for the production of one well defined (in duration and amplitude) square-wave pulse for each oscilloscopic sweep for calibration purposes.

The chlorided silver wire inside the recording microelectrode was connected to the input of a unity gain cathode follower described by Bak (3). The output of the cathode follower was connected to a Tektronix type 502A dual-beam oscilloscope. (Oscilloscopic photographs were taken by a Grass Model C-4 camera which was attached to a Tektronix 502 oscilloscope modified to function as a "slave" scope).

The unity gain cathode follower described by Bak (3) was used in a slightly modified circuit. As shown in Figure 28, the potentiometer R_1 was introduced in parallel to the potentiometer R_2 in order to achieve quickly and conveniently a D.C. output at ground level. The output cable was doubly-shielded with the inner shielding connected to the cathode of the input tube and the outer shielding connected to ground. A Mullard type EF 86 pentode was used instead of a Z 729 pentode. Furthermore, the voltage B_3 was increased from 110 V to 112.5 V. Actual circuit components were as follows:

V_1 EF 86 (Mullard), V_2 12 AU 7 (RCA), C_1 1.5 to 7.0 $\mu\mu$ F, C_2 250 $\mu\mu$ F,

R_1 50 K pot., R_2 10 K pot., R_3 75 K 1W, R_4 390 K, R_5 5 K pot.,

R_6 33 K 1W, B_1 45 V, B_2 45 V, B_3 112.5 V.

Figure 29 demonstrates the electrical characteristics of the modified cathode follower. A square-wave test pulse of 1.5 msec duration and 30 mV amplitude was introduced into the input in series with a 600 Megohm resistor. Fine adjustment at unity gain and zero grid current was done by varying the capacitor C_1. Figure 29 A and B show an under-compensated and an over-compensated output test pulse, respectively. In Figure 29 C optimal adjustment is illustrated with no appreciable distortion of the output test pulse. (Figure 29 D

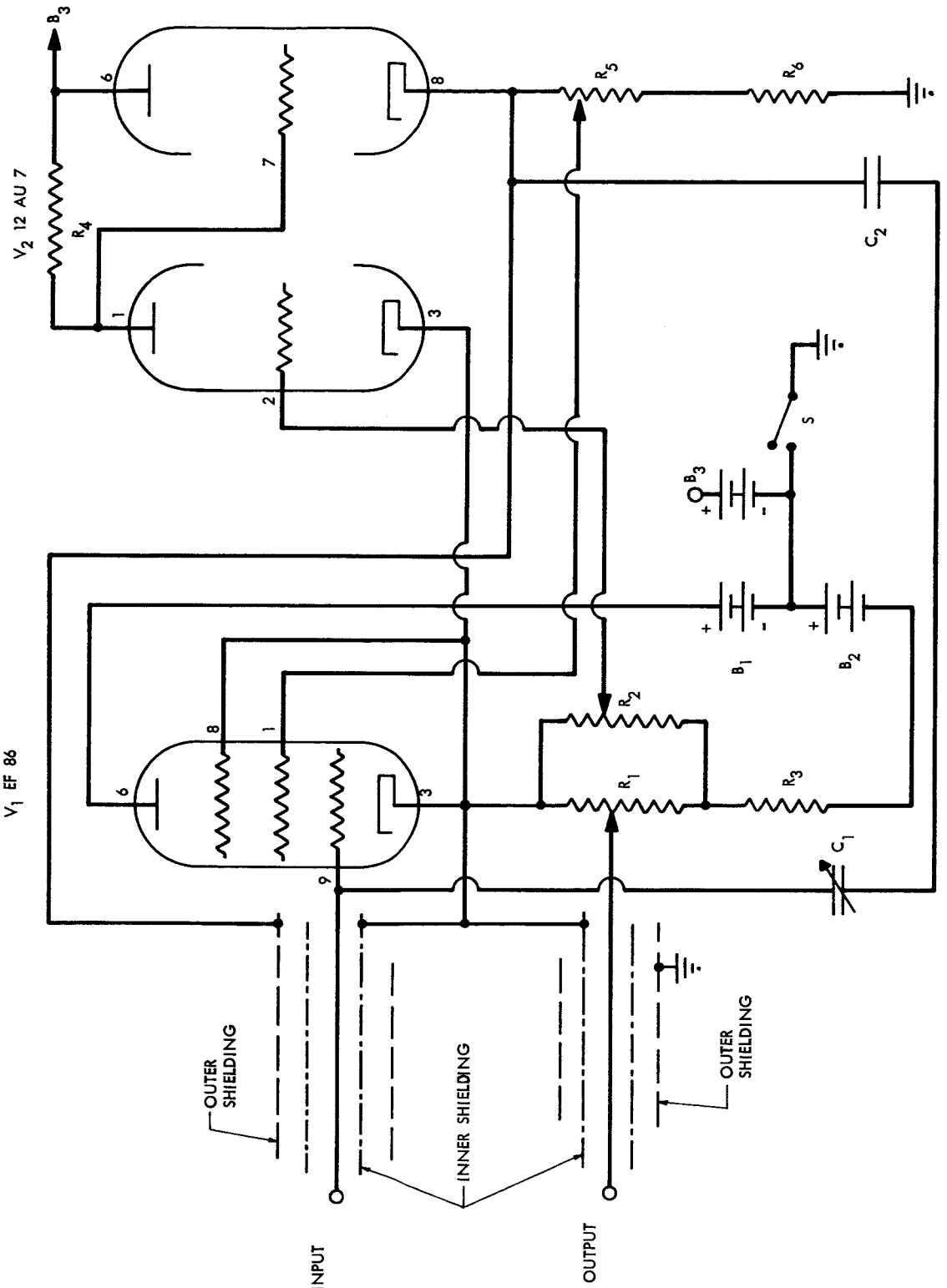

FIGURE 28. - UNITY GAIN AMPLIFIER CIRCUIT OF A. F. BAK, SLIGHTLY MODIFIED

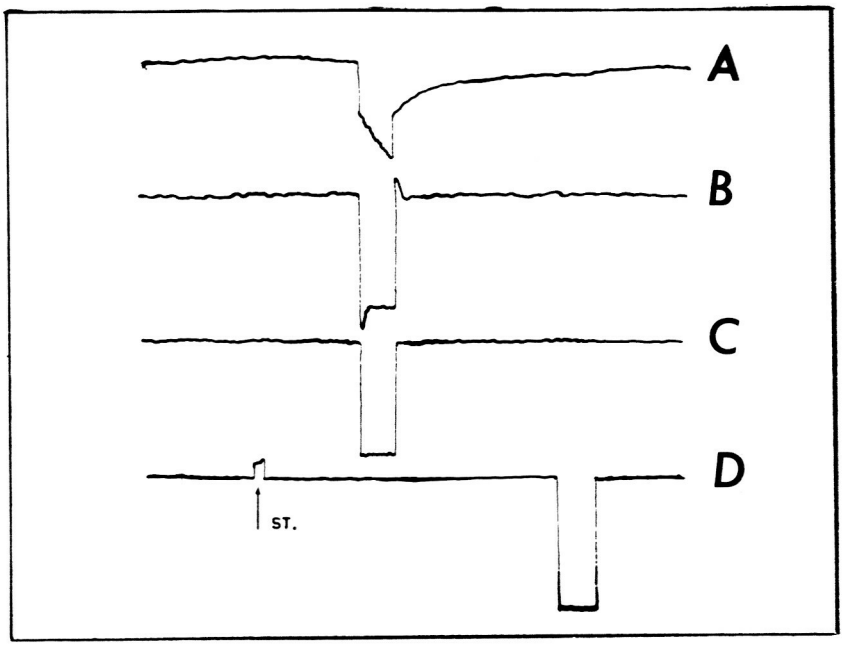

Figure 29. Calibration pulses (30 mV; 1.5 msec) as recorded in series with a 600 Megohm resistor, for details see text.

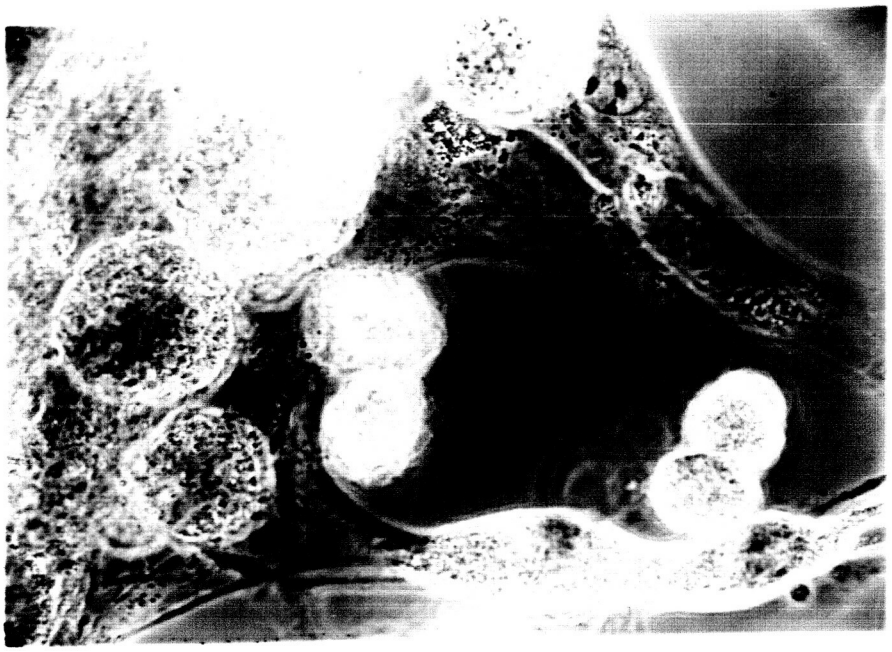

Figure 30. Ten day old culture of cerebellum showing explant and outgrowth.

shows a calibration pulse with a preceding small stimulus artifact. This figure will be described later).

All tissue culture experiments were carried out at a mean temperature of 26°C.

3. Technological Accomplishments

Plexiglass Recording Chamber:

Plexiglass chambers to hold the coverslip containing the explant during experimentation were designed and constructed by the investigators. These chambers differed from those described by Hild, Chang, and Tasaki (22) in that the width of the chamber floor was narrower and the space between the chamber roof and the chamber floor was smaller. In addition, a U-shaped filling and grounding glass tube (see Figure 27) was introduced, permitting rapid exchange of electrolyte solution in the recording chamber. With the exception of the glass chamber floor and the glass filling tube all parts of the chamber were made of plexiglass. The floor measured 8 mm x 25 mm and was permanently glued to the plexiglass body with Epoxy cement. The exchangeable filling tube of 5 mm I.D. fitted neatly in the rear of the chamber.

Part of the chamber described is shown in Figure 25; the glass floor is positioned over the microscope condenser. The rear and front ends of the chamber are connected by two plexiglass stabilizing plates. In the upper part of the picture the duct for the filling tube can be seen.

In order to make all surfaces (particularly the Epoxy joints) biologically inert, smooth, and scratch-resistant, the entire chamber was treated with a water soluble silicone solution (SILICLAD*). Since properly applied silicone layers are characterized by a high degree of stability, the coating of the chambers withstood numerous cleaning procedures before it had to be renewed.

*SILICLAD is available from Clay-Adams, Inc., New York 10, N. Y.

Microelectrodes:

Metal-filled glass pipette microelectrodes which were used for extracellular stimulation in tissue culture are described in detail in the "Appendix". Using a bipolar stimulating arrangement with a small cathodal and a larger anodal electrode (See diagram in Figure 27) it was possible to reduce the stimulus artifact to a very low level. Figure 29 D shows a stimulus artifact (St. ↑) recorded under experimental conditions through a 40 Megohm hyperfine glass pipette. Although a strong square wave stimulus shock (6 V amplitude; 0.5 msec duration) was applied to the stimulating electrode, and although the tip of the recording electrode was positioned within a few microns of the small cathodal stimulating electrode, the recorded artifact was small and measured 4 mV in amplitude. There was no distortion of the baseline which so often is seen with the use of unipolar and grounded stimulating arrangements. (The calibration pulse in Figure 29 D is a positive pulse of 30 mV amplitude and 1.5 msec duration). A view of the bipolar stimulating arrangement is given in Figure 25; a fine "different" and a larger "indifferent" electrode were introduced as a pair into the recording chamber from the right side. A hyperfine glass recording microelectrode is shown at the left side.

Optical Split Image System:

It is of great importance that at every instant the experimenter be fully aware of the occurrence of bioelectrical activity associated with fine movements of the recording microelectrode as seen through the microscope. For this reason an optical system was designed and built by the investigators enabling the observer to view simultaneously the microscopic field and the bioelectrical patterns displayed on the oscilloscopic screen. This equipment made possible the determination of precise location and instant at which bioelectrical activity occurred. In these arrangements, the experimenter viewed the microscopic field with his right eye, while his left eye saw the

image of the oscilloscopic recording screen through a half binocular (X 7), mounted 65 mm toward the left of the left microscope eyepiece. The arrangement described together with one of the reflecting mirrors is shown in Figure 26.

4. Experimental Results

Morphological Observations:

Utilizing the "flying coverslip" method of culturing nervous tissue explants, outgrowth containing cellular components of the central nervous system was obtained. Outgrowth was observed from original explants of cerebellum, superior colliculus, and dorsal root ganglia. Cultures of dorsal root ganglia produced abundant bipolar neurons. Large Purkinje cells surrounded by a dense network of glial cells were found in 10 to 12 day old cultures of rat cerebellum. Outgrowth from explants of white matter (corpus callosum) of 10 to 11 day old rats contained cellular elements tentatively identified by phase-contrast microscopy as neuroglia. Figure 30 shows cellular elements in the outgrowth zone of a 10 day old culture of cerebellum. A typical outgrowth of neuronal network in a 12 day old culture of dorsal root ganglia (rat) is demonstrated in Figure 31.

Following 4 weeks in culture no significant differences in morphological integrity were observed between cultures of dorsal root ganglia containing 300 mg% to 3,000 mg% of glucose. However, in the fifth week, extensive flattening and granular degeneration with gradual loss of cellular outlines was observed in all cultures containing the various glucose concentrations.

For recording purposes 10 to 12 day old cultures were used. After 15 to 18 days, cellular elements were flattened to such an extent that penetration with the recording microelectrode was almost impossible.

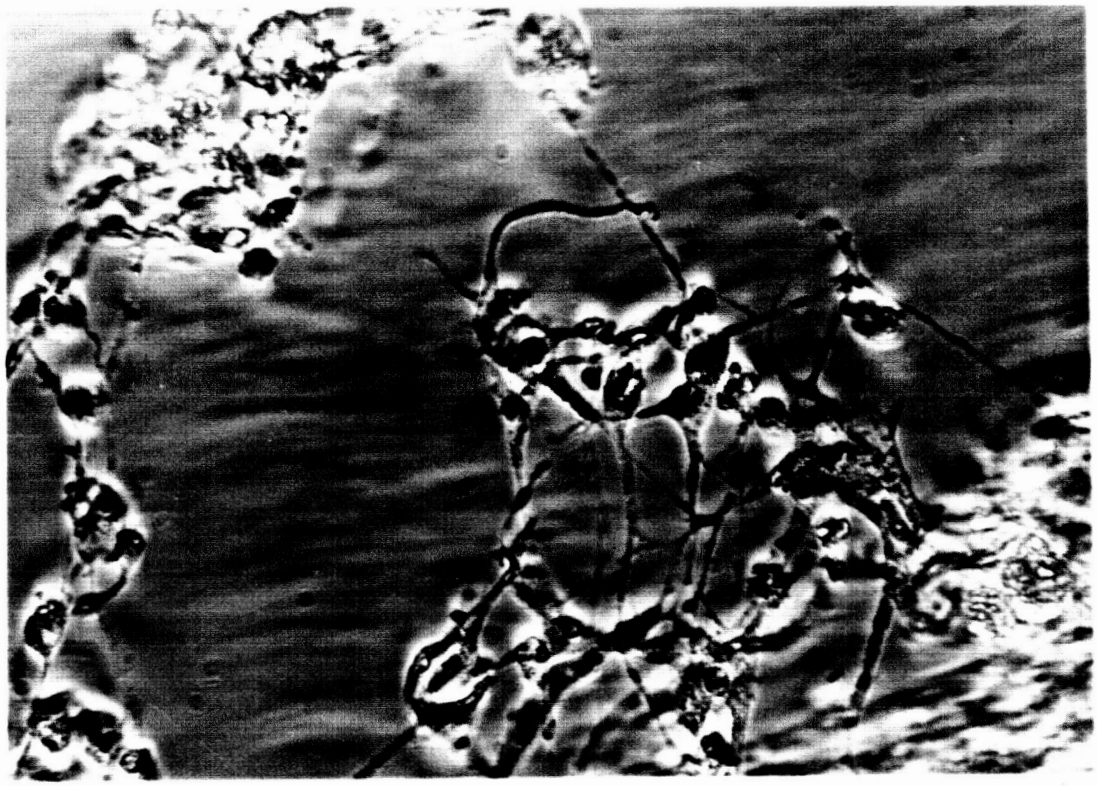

Figure 31. Outgrowth of neuronal network (dorsal root ganglion of rat; 12 days) as visualized through the phase-contrast microscope of the micromanipulator. X 400.

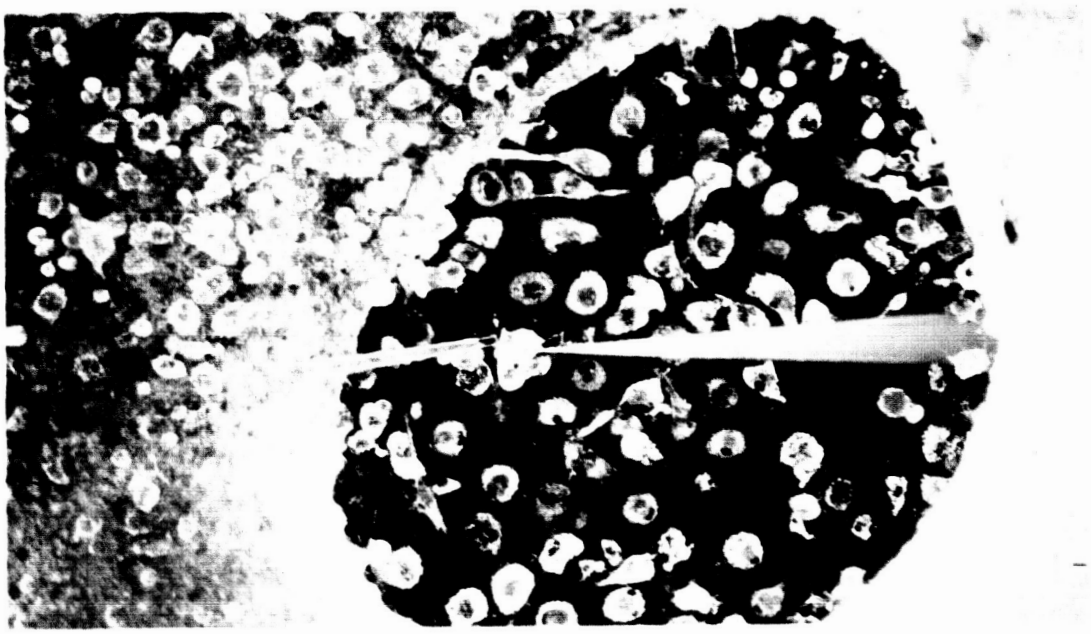

Figure 32. Recording and stimulating electrode positioned in the vicinity of a cell in a halo of thin outgrowth. X 120.

Electrophysiological Findings:

During electrophysiological experimentation with neurons and neuroglial cells in tissue culture photographs of the microscopic field were taken frequently. Typical views of recording and stimulating microelectrodes in a 12 day old culture of cerebellum are presented in Figures 32 and 33: Figure 32 shows a hyperfine recording microelectrode (right) and a fine (5 μ) metal stimulating electrode (left) positioned in the vicinity of a cell in a halo of thin outgrowth (X 120). Figure 33 is a photograph at higher magnification (X 480). It shows a neuron at the edge of a dense outgrowth zone. The hyperfine recording electrode (right) is in close contact with the cell body. The tip of a metal stimulating electrode (left) is located in the vicinity of the arborizations of the dendritic tree.

Since both electrodes are approaching the cell at an angle of approximately 30° from below, only the electrode tips are in focus. The larger "indifferent" stimulating electrode of the bipolar stimulating arrangement is not within the visible microscopic field of Figure 32 and 33.

More than 50 cultures of dorsal root ganglia and cerebellum were examined in the tissue culture chamber, and attempts were made to record bioelectrical parameters from neurons and neuroglial cells. It was difficult to impale cells in monolayer outgrowth zones since these cells were not attached firmly enough to their surroundings, and distortion of the dendritic network was almost inevitable. On the other hand, cells located in denser outgrowth areas were more difficult to approach. However, impalement of the cells was easier in the three-dimensional network.

Figure 34 shows the oscilloscopic tracings of D. C. shifts obtained after penetration of a neuron (A; 65 mV) and a neuroglial cell (B; 80 mV). After withdrawl of the hyperfine recording electrode (after approximately 10 sec.) the oscilloscopic beam returned

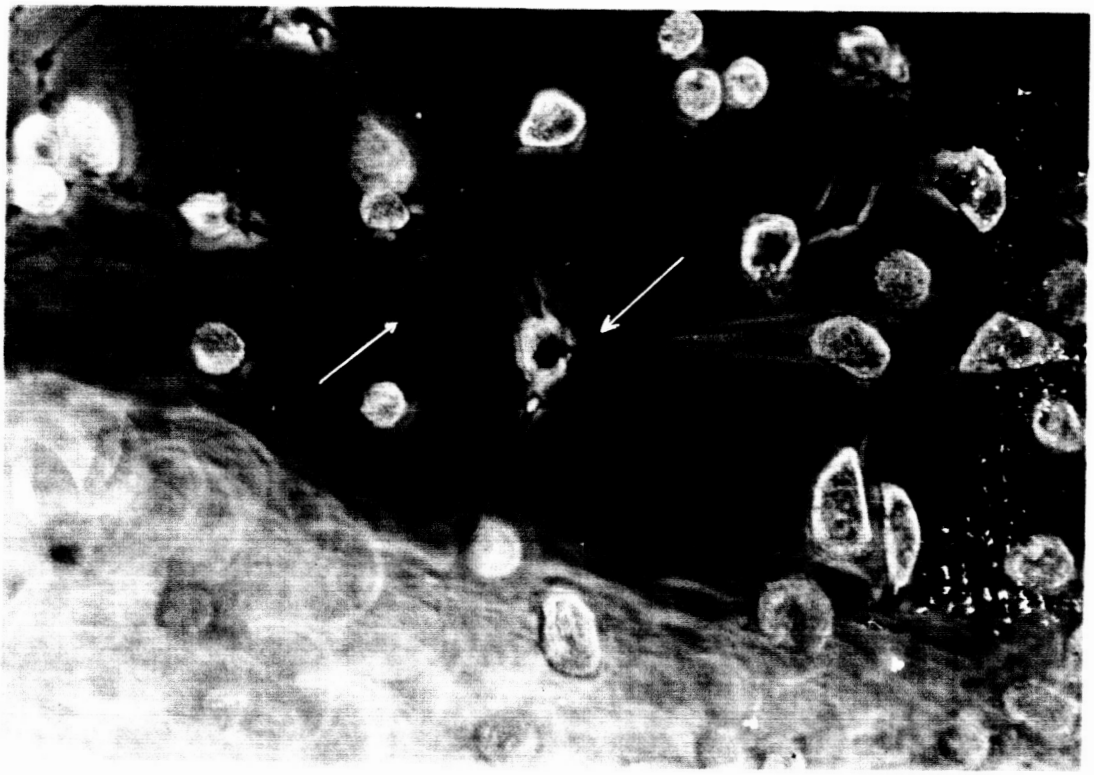

Figure 33. Neurons at the edge of a dense outgrowth zone. Hyperfine recording electrode (right) and stimulating electrode (left) in close contact with the cell. X 480.

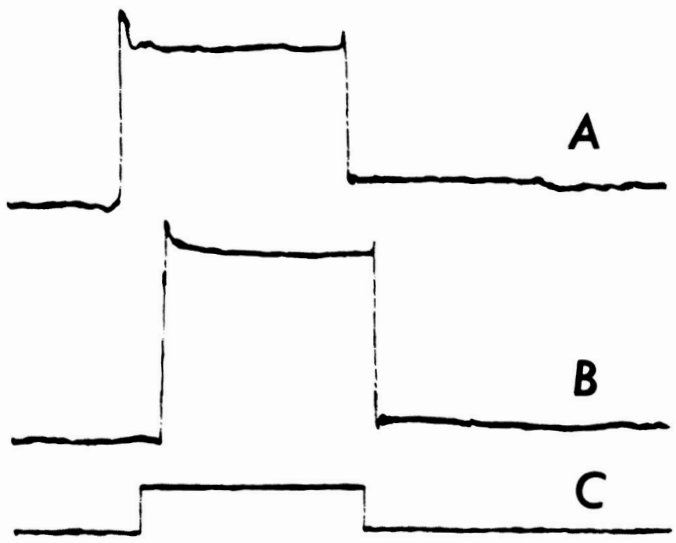

Figure 34. Oscilloscopic tracings of D. C. shifts obtained after penetration of a neuron (A; 65 mV) and a glial cell (B; 80 mV). C: Calibration pulse (20 mV; 10 sec).

to the baseline level.

It was difficult to eliminate the transfer of small involuntary hand and finger movements through the micromanipulator. This deficiency made hyperfine electrode movements impossible, particularly when both stimulating and recording electrode were located in the immediate vicinity of the cell. The limitations in manual dexterity and precision during extremely small finger and hand motions, in combination with the characteristics of the micromanipulator, were found to constitute an experimentally inadequate means for obtaining with reasonable efficiency recordings of evoked action potentials. Intracellularly evoked action potential were, however, clearly observed a number of times on the oscilloscope screen, but photographs were not successfully obtained of these transient occurrences. On a few occasions, spontaneous extracellularly recorded action potentials of approximately 0.5 mV amplitude were derived from neurons in tissue culture.

After elimination of the described mechanical deficiencies, the stimulating and recording system used should represent a satisfactory tool for further neurophysiological and neuropatho-physiological studies in tissue culture.

Biochemical Findings:

In addition to the morphological and electrophysiological findings, an amino acid analysis of the medium surrounding a 3 day-old culture of cerebellum was performed which revealed the interesting fact that the level of glutamic acid was decreased whereas that of proline was increased. Control media incubated for 3 days did not show significant changes in proline and glutamic acid as compared to fresh unused media. Quantification of the chromatographic analysis at 570 mμ revealed the following amino acid concentrations:

	Control (in μM)	Cultures (μM)
GLUTAMIC ACID	2.42	0.57
PROLINE	0.50	1.14

The significant portion of the chromatogram of the amino acid analysis of medium from a 3 day old explant culture of cerebellum compared to fresh unused medium as control is shown in Figure 35.

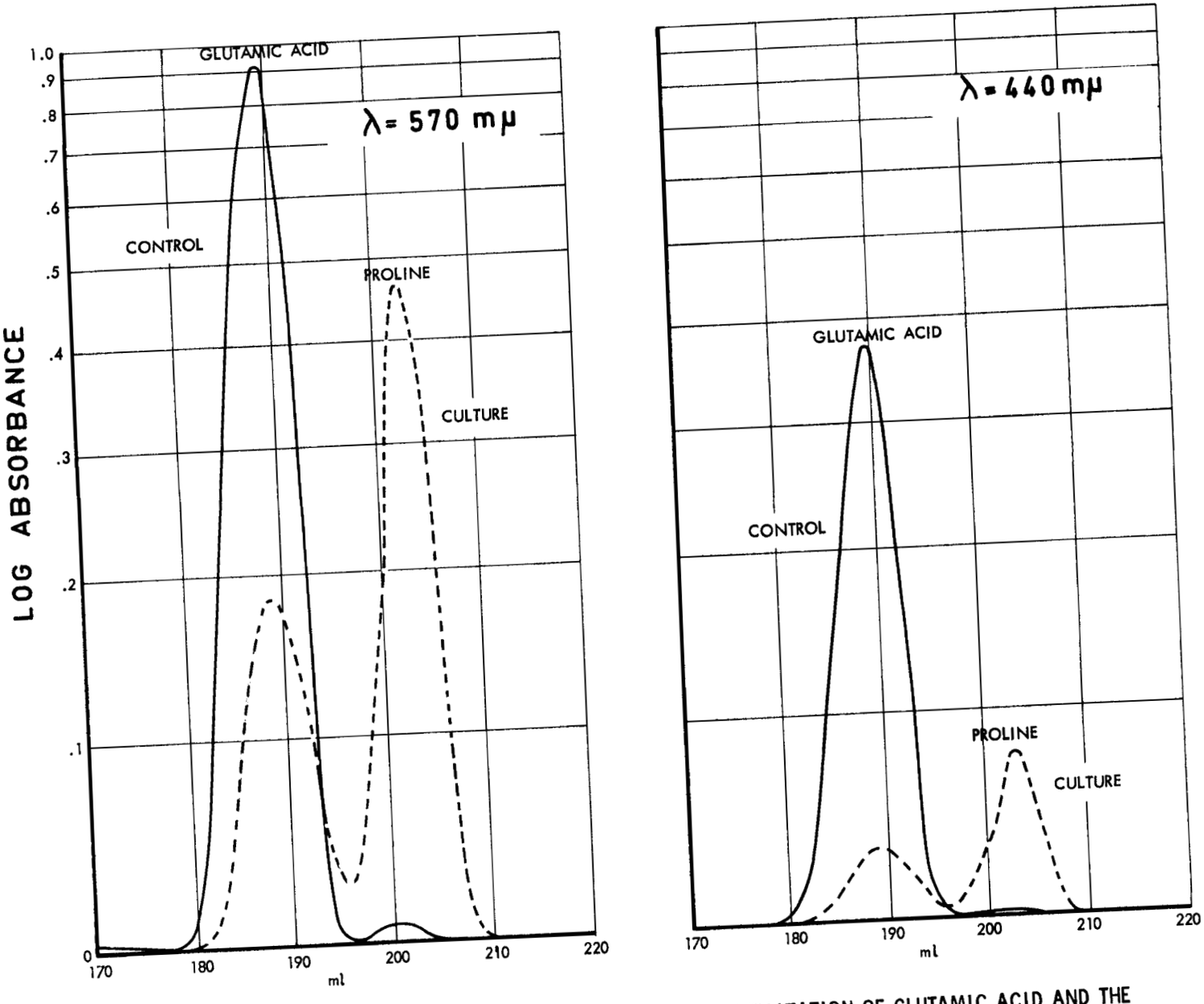

FIGURE 35. - AN AMINO ACID ANALYSIS DEMONSTRATING THE UTILIZATION OF GLUTAMIC ACID AND THE EXCRETION OF PROLINE BY CEREBELLUM EXPLANTS FOLLOWING THREE DAYS IN CULTURE COMPARED TO FRESH UNUSED MEDIUM AS CONTROL

IV. DISCUSSION

In this investigation of the effects of ionizing radiation on the central nervous system in vivo a "high voltage slow wave syndrome" was observed following 4,000 r and 2,000 r head X-irradiation. This effect has been described earlier by Eldred and Trowbridge (9), Ross, Leavitt, Holst, and Clemente (35), Brooks (4), and Rube (36). These authors agreed that this slowing of EEG activity occurred within 1 to 4 days following irradiation. Monnier and Krupp (31) reported the development of a "high voltage slow wave syndrome" with numerous spindles in the rabbit as early as 30 minutes after exposure to 900 r.

In this investigation a gradual loss of cerebral cortical and hippocampal arousal pattern was observed after a short initial period of enhancement. This observation is in agreement with Gangloff and Haley (15) who reported a decreased reticular arousal threshold in animals exposed to 400 r WBR.

Hippocampal spiking after head irradiation was also reported by Gangloff (13), Gangloff and Haley (15), and by Monnier and Krupp (31) who observed marked activation of the archicortex with spike potentials after exposure to 400 r. The fact that after exposure to 4,000 r the hippocampal leads became almost isoelectric indicates the relative radiosensitivity of this structure in comparison with other regions from which recordings were made.

A comparison of cortical and subcortical electrical activity indicated strongly that cortical post-irradiation effects appear later and secondary to subcortical effects. This finding is very reminiscent of the observations made in human patients during the development of an epileptic seizure (Penfield (32)). Ribstein (34)

described a grand mal case with primary spontaneous spiking activity in the hippocampus. In another patient with temporal lobe epilepsy the same author observed a spontaneous electrical crisis of the amygdala. Based on experiments by Jung (27) and Gastaut and Hunter (19), Gastaut and Fischer-Williams (18) suggested that generalized discharges originate in diencephalic structures from where they radiate to the whole brain. Our own findings as well as the conclusions of Gastaut and Fischer-Williams (18) are not in agreement with observations by Starzl et al.(40) who in their studies with Metrazol induced seizures reported the cerebral cortex to be primarily involved in seizure activity.

We found a lowered seizure threshold in our head X-irradiated animals. Similar observations were reported earlier by Andrews (2), Schwartzbaum et al (38), and others.

After 2,000 r head X-irradiation, preliminary impedance measurements in the hippocampus revealed an immediate and significant drop in impedance. Adey, Kado, and Didio (1) demonstrated that arousal from normal sleep was also followed by a baseline shift towards a lower impedance. Since the initial phases following irradiation are accompanied by a typical arousal pattern, it is not surprising to see a drop in resistance and capacitance during that period.

Later developments were marked by a significant instability of the impedance baseline. This observation is in agreement with Schoenbrun, Campeau, and Adey (37), who observed large impedance baseline excursions in the hippocampus after head irradiation.

It could be speculated that the impedance excursions in the brain after irradiation reflect the metabolic instability of the central nervous system as a response to the radiation stress. It is possible that the method of impedance measurements constitutes a sensitive indicator for early alterations in the central nervous system, long before other bioelectric abnormalties can be detected.

For many years the adult nervous system was thought to be relatively radioresistant. More recent studies indicate that low doses could cause subtle changes in the brain. In view of reports claiming low dose effects (Grigoryev (20): Lebedinsky et al. (28); Miller (30); Garcia et al. (16;17) Hug (24); and Hunt and Kimeldorf (25)) it is suggested that further studies, including pre- and post-irradiation impedance measurements, should be carried out with gradually decreasing doses of ionizing radiation.

The second research objective of this program was the investigation of radiation effects on the central nervous system at the cellular level. Substantial effort was devoted to an attempt to establish a satisfactory experimental approach for such an investigation.

Concentrations of glucose up to 3,000 mg% were observed not to sustain the morphological integrity of neurons in tissue culture to any greater extent than 300 mg%. The role glucose plays in the enhancement of neuronal survival, glial viability, and myelin formation is obscure. Glucose is an important energy source for the cell, but this may not be its primary mode of action in promoting survival of cells in tissue culture.

An amino acid analysis of the medium from an explant culture of cerebellum compared to fresh unused medium indicated that after 3 days in culture glutamic acid was metabolized and proline was accumulated in relatively large amounts. These observations are preliminary but merit reporting.

More detailed and extensive biochemical studies are necessary before coming to valid conclusions regarding possible glutamine-proline metabolic interactions in the central nervous system under tissue culture conditions. The role of glutamic acid in the nervous system has been discussed by Weil-Malherbe (45), and recently, by D. Tower (47).

The fact that D. C. shifts up to 80 mV could be recorded from neurons and neuroglial cells in tissue culture indicates the viability of the test cultures. Typical spontaneous extracellular action potentials and evoked intracellular action potentials were observed on the oscilloscopic screen. Such observations were clear although transient in nature. These observations are considered to be an indication that normal electrical activity existed in the cerebellar and spinal cord tissue cultures under test.

Reproduction of the findings of Hild and Tasaki (23) was not attained in the first research year of the contract. The intense effort made to record evoked action potentials from neurons in culture has even made more clear to the investigators the great skill and patience exhibited by Hild and Tasaki in their pioneering achievement. We are also convinced that an instrumentation development effort is required in order to expedite research progress in this area. We hold further that greatly improved micromanipulative and recording techniques are feasible within the present state of the instrumentation art. At present the limitations in finger and hand dexterity and steadiness are severe handicaps to the efficient and precise positioning of the microelectrodes; the hours of continuous microscopic inspection required constitute a severe visual strain on the investigator. The elasticity and mobility of the cells tend to make proper electrical contact difficult to attain and difficult to maintain beyond a few seconds. If research is to progress rapidly in this very important field, with measurements and recordings to be made from networks of cells, improved instrumentation is an absolute necessity. With reasonable and feasible instrumentation development, tissue culture is believed to possess an excellent potential for electrophysiological examinations of single cells and of interconnected small groups of cells.

V. SUMMARY

The investigations of this program of research consisted in two principal efforts: the first was devoted to studies of the effects of ionizing radiations on the central nervous system in vivo; the second was directed toward exploration of the effects of ionizing radiations at the cellular level.

In the research in vivo, the effects of ionizing radiations on the central nervous system of cats was investigated using deeply implanted brain electrodes as a means of studying bioelectrical activity before and after irradiation. The recording techniques and the interesting results of impedance measurements in nervous tissue are described.

In cats exposed to 4,000 r and 2,000 r head X-irradiation, a profound effect on the cerebral cortical activity was seen in the form of a "high voltage slow wave syndrome". Subcortical areas such as the hippocampus and the amygdaloid nuclei showed spontaneous spiking and at higher doses (4,000 r) gradually became isoelectric. A significant lowering of the seizure threshold also appeared, and cortical post-irradiation effects seemed to appear later and secondary to subcortical effects.

Measurements in the hippocampus showed an immediate drop in impedance magnitude after irradiation followed by extensive excursions in the magnitude of resistive and reactive components and in their ratio.

These various results are discussed in relation to literature findings. The obvious desirability of continuing the investigations is emphasized in a recommendation for further studies at gradually decreasing exposure doses.

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IX. A P P E N D I X

An Evaluation of Microelectrodes

for

Stimulation in Tissue Culture

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IX. APPENDIX*

An Evaluation of Microelectrodes for Stimulation in Tissue Culture

Intracellular Stimulation:

If the delivery of electrical currents through intracellular microelectrodes is intended, double-barreled microelectrodes (Coombs, Eccles, and Fatt (4)) or single microelectrodes in connection with a bridge circuit (Araki and Otani (1); Frank and Fuortes (9)) can be used. An approximate threshold intensity of 7×10^{-8} Amp. (14) can be reached with hyperfine intracellular glass pipettes without the electrode markedly departing from pure (ohmic) resistance (8).

Extracellular Stimulation:

Extracellular stimulation has important application where it is intended to stimulate and to record at different sites, for example: recording complex bioelectric activity in explants (5), demonstrating the propagation of bioelectric impulses along certain structures, recording a so-called "glial response" (13; 14; 17) etc.

Preferably microelectrodes used for extracellular stimulation in tissue culture should have tip diameters not exceeding 2 to 10 μ in order to make possible close contact with a well defined area of the excitable structure. Secondly, the stimulating electrodes should be non-polarizable in order to avoid a disturbance of the ionic equilibrium condition, characterized, for example, by the formation of gas on the effective electrode tip surface with a consequent change of electrode resistance. Thirdly, a low impedance of the stimulating electrode is desirable. High impedance microelectrodes require the application of a relatively high voltage stimulation pulse in order to insure sufficient current density at the electrode tip near the excitable structure. For example, in order to excite the cell soma of a neuron with short shocks of approximately 20 μ A at threshold using a 10 μ BSS Gey-agar gel filled glass pipette of approximately

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*The abbreviation "app." in connection with figures and as used in the text stands for "appendix".

1.5 Megohm resistance (14), one would have to apply stimulus pulses of at least 30 V amplitude to the electrode. Application of relatively high pulses, however, gives rise to unduly high and distorting stimulus artifacts, and, if the decomposition voltage of the electrode-elecrolyte system is exceeded (e.g. over 1.7 V with smooth platinum in aqueous solution (10)), irreversible and undesirable processes take place at the effective electrode tips.

In search for an ideal stimulating electrode for extracellular experimentation in tissue culture, we tested and evaluated various microelectrode configurations and structures as follows:

a) Crain and Peterson (5) and Wardell (17) used saline-filled glass pipettes with tip diameters up to 15 to 20 μ, respectively. Hild and Tasaki (14) and Hild, Chang, and Tasaki (13) employed glass microelectrodes (5 u to 15 u) filled with BSS Gey-agar gel or Gey's balanced salt solution. According to Hild and Tasaki (14) electrodes of about 10 μ in tip diameter had a resistance in the range of 1.2 to 1.5 Megohm. Our own measurements with 0.9 % NaCl filled glass pipette electrodes revealed higher values (see Figure 1 app.). Inner tip diameters of 10 μ and 15 μ were found to correspond to electrode resistances of 3.8 and 1.5 Megohm, respectively. However, the differences between the values of electrode resistance obtained by us and Hild and Tasaki may well be accounted for by a different cone semi-angle of the tested electrodes.

In order not to exhaust the capacity of an average stimulating system (50 V shocks via stimulation unit) and to provide shocks of at least double threshold strength (approximately 40 μ A for neuron soma in tissue culture; (14)), the resistance of a saline-filled glass pipette should not exceed 1.25 Megohm, i. e. the inner tip diameter should be at least 16 μ. These conditions apply to unipolar stimulation only.

We conclude that extracellular glass stimulating electrodes with

normal saline or Ringer's solution as conductive cores have the disadvantage of high resistance with a small tip diameter or of relative bulkiness at acceptable resistance levels.

b) If the conductivity of normal saline is compared with that of platinum, for example, (3) it is apparent that platinum conducts electricity approximately 5.6×10^6 times better than normal saline. For this reason we attempted to produce a platinum microelectrode by coating a glass blank of desired shape with metallic platinum, and then insulating the electrode with the exception of the tip. Liquid Bright Platinum # 05-X (Engelhard Industries, Inc., (7)) was used as a coating on fabricated glass blanks and processed properly. The resulting products were glass cones with tip diameters of 1 to 10 μ, covered with a thin layer (approx. 0.2 μ) of bright platinum. Under microscopic view (X 600) the metallic layer seemed to be very smooth and homogeneous. Calculations indicated that the microelectrode resistance should not have exceeded 2 Kilo-ohms. Our resistance measurements, however, revealed very high values, for example in excess of 15 Megohms for tip diameters of 5 μ. This fact could possibly be explained by the existence of hyperfine cracks in the platinum coating (2). Other metal depositions have not been tested by us as coatings. It is possible that gold deposits on glass blanks would constitute a more successful approach.

c) Stainless steel electrodes successfully used for recording purposes (Hubel (15); Grundfest, Sengstaken and Oettinger (12); Green (11)) are unsuitable for stimulation because of polarization problems and the fact that toxic heavy metal ions are introduced into the tissue culture chamber during current flow.

We remedied these disadvantages by electroplating successive layers of copper, gold, platinum and platinum black on electrolytically pointed hyperfine steel needles. With the exception of the very tip the electrodes then were insulated with five layers of Epoxylite

according to the specifications of the manufacturer. Although the resulting electrodes were non-polarizable, very small in size, and biologically inert, the insulation was not satisfactory : porosity of the insulation could not be completely eliminated; the capacitance of the electrode against the surrounding electrolyte solution was too high (in excess of 50 $\mu\mu$F per mm electrode length) and led to a distortion of the square wave stimulating pulse; the cells of the tissue culture had a tendency to stick firmly to the Epoxylite surface and to form a bulky layer around it.

The finding of an insulating coating for hyperfine metal needle electrodes which will overcome the disadvantages mentioned remains a problem.

d) Svaetichin (16) and Dowben and Rose (6) have devised low impedance microelectrodes by filling fine glass pipettes with metal. Although these investigators have used their metal-filled glass microelectrodes for recording purposes, it seems to us that a slightly modified type of electrode could be successfully employed for stimulation in tissue culture. We have modified the method of Dowben and Rose (6) and produced glass microelectrodes with platinum endings varying from 2 to 10 μ in the smaller range for "different" electrodes, and up to 80 μ in the larger range for "indifferent" electrodes in a bipolar stimulating arrangement.

Indium of highest purity (American Smelting and Refining Co) was melted in a large test tube which in turn was submersed in a mineral oil bath heated to 220°C on a combination hot plate and magnetic stirrer. Thoroughly cleaned glass capillary tubing (length 150 mm; O.D. 1.2 mm) was introduced into the molten metal and by means of gentle suction (using a syringe) the indium was drawn up as a continuous column to approximately the midpoint. Two to four mm beyond the end of the indium column a fine glass tip was drawn. Figure 3 app. demonstrates seven stages in preparation of a metal-filled glass microelectrode with an inner tip diameter of 2 μ. A fine glass pipette (B) was either drawn directly or obtained by careful breakage of a hyperfine glass tip (A) under microscopic inspection. As shown in Figure 4 app. the incompletely filled pipette was held to a preheated hot plate (550° - 600°F) in a vertical position. A No. 23 gauge injection needle was introduced slowly and completely into the blunt end of the pipette. A syringe may be attached to the needle for better manual control. The pipette was repeatedly heated and then allowed to cool. During each exposure to heat the indium core inside the micropipette progressed for a short distance down toward the tip without external pressure on the column. Finally, the molten indium reached the tip and formed a minute metal ball (C in Figure 3 app.). The metal ball was gently blown off. Undesirable remainders of the indium ball or a cuff of indium formed on the outer surface of the glass pipette can be successfully removed by dipping the electrode in concentrated sulfuric acid for 10 to 30 seconds. The resulting completely filled micropipette is shown in D of Figure 3 app.

Dowben and Rose coated the tips of their microelectrodes with a layer of gold immediately after the filling operation. We found that greater mechanical stability is achieved if the coating of the indium is carried out inside the micropipette. Therefore, under continuous microscopic inspection a few microns of the indium core were electrolytically dissolved in a solution of 20% to 30% ferric nitrate

$[Fe(NO_3)_3]$. The current necessary for electrolytic dissolution is very low. We used a 3 V.D.C. source in series with a 44 Megohm resistor for 2 μ pipettes, lower resistors for larger size pipettes. The indium filled pipette was made the anode. The cathode was a platinum wire dipped into the ferric nitrate solution. After only a few seconds a complete dissolution of a few microns of the indium core was obtained (E in Figure 3 app.).

The electrode was then dipped into distilled water for 10 minutes or longer to allow removal of indium salts and the remaining ferric nitrate.

A silver cyanide solution (see below) was used for plating the reduced column of indium. The micropipette was made the cathode and the anode was a pure silver wire. A 3 V.D. C. source in series with a 44 Megohm resistor provided the plating current for 2 μ pipettes. Electrodes with larger diameters required lower resistors (e.g. 1 Megohm for a 60 μ tip) in series with the source. F of Fig. 3 app. shows micropipette after electrolytic deposition (for 20 seconds) of approximately 5 μ of silver on the reduced indium core.

After rinsing in distilled water a final layer of platinum (using weak plating currents) and then platinum black (using stronger plating currents) was electrodeposited on the silver. A solution of 0.4 % chloroplatinic acid was used. The plating current was somewhat stronger than that used for silver plating. The final microelectrode is shown in G of Figure 3 app. With its attached injection needle it can be quickly and conveniently connected to plugs and micromanipulator holding devices.

All plating processes were conducted with continuous visual inspection through a microscope. The plating solutions were kept in a small chamber into which the indium pipette was introduced.

It is important to mention that the water for rinsing purposes

should not significantly exceed room temperature. Otherwise thermal shock causes an interruption of the column continuity at the very tip and leads to destruction of the electrode.

The electrodes described here differ from those described by Dowben and Rose in that these investigators used gold to plate the indium. In numerous tests with macroscopic specimens of indium we found that gold electrodeposited from a gold cyanide solution does not adhere to indium as a "chemically inert and impervious gold base" (6). On the contrary, it is deposited in a finely divided form and can be easily wiped off. On the other hand, we found that successive coatings of indium with copper (from a copper cyanide solution) and gold (from a gold cyanide solution) gave excellent results. In our first metal-filled microelectrodes we used this approach.

Further tests with a large piece of indium indicated that electrodeposited silver formed an excellent and mechanically sturdy coating on indium. It was therefore decided to use an impervious silver base in the production of metal-filled microelectrodes. The following silver plating solution was used:

	g / l
Silver Cyanide, AgCN	17.0
Potassium Cyanide, KCN	26.7
Sodium Carbonate, $NaCO_3$	40.0
Thiourea, $(NH_2)_2$ CS	0.001

All steps employed in the production of our microelectrodes were tested with macroscopic indium specimens; this includes electrolytic dissolution of indium in a solution of ferric nitrate and subsequent electrodeposits of copper, gold, and platinum, or silver, platinum, and platinum black.

The metal-filled stimulation microelectrodes described offer numerous advantages as follows:

1. In spite of the small tip diameter, the electrode impedance

is comparatively low. In Figure 2 app. the relationship of tip diameter and electrode impedance is shown in contrast to a similar graph (Figure 1 app.) for 0.9% NaCl-filled micropipettes.

2. Due to the final platinum black layer the electrodes are practically non-polarizable. In our quality tests carried out in Ringer's solution, gas bubble formation occurred at the tips of cathodal microelectrodes if the voltage of the stimulation pulses (10 msec duration; 20 per second) exceeded 6.4 V. However, the pulse voltage necessary to insure sufficient current density for stimulation at the cellular level is significantly lower, for example approximately 1.4 V at threshold level for a 5 μ microelectrode.

3. The capacitance of metal-filled glass microelectrodes against the surrounding grounded electrolyte solution is very low (not exceeding 0.5 μμ F per mm length) and does not interfere appreciably with the shape of the square wave stimulation pulse.

4. The described stimulation microelectrodes are re-usable.

5. The glass insulation of the metal microelectrodes is very smooth so there is little tendency for cellular elements to stick to the electrode surface. In addition, by treating the finished electrode with a non-toxic water soluble silicone solution (SILICLAD) the electrode surface can be further improved.

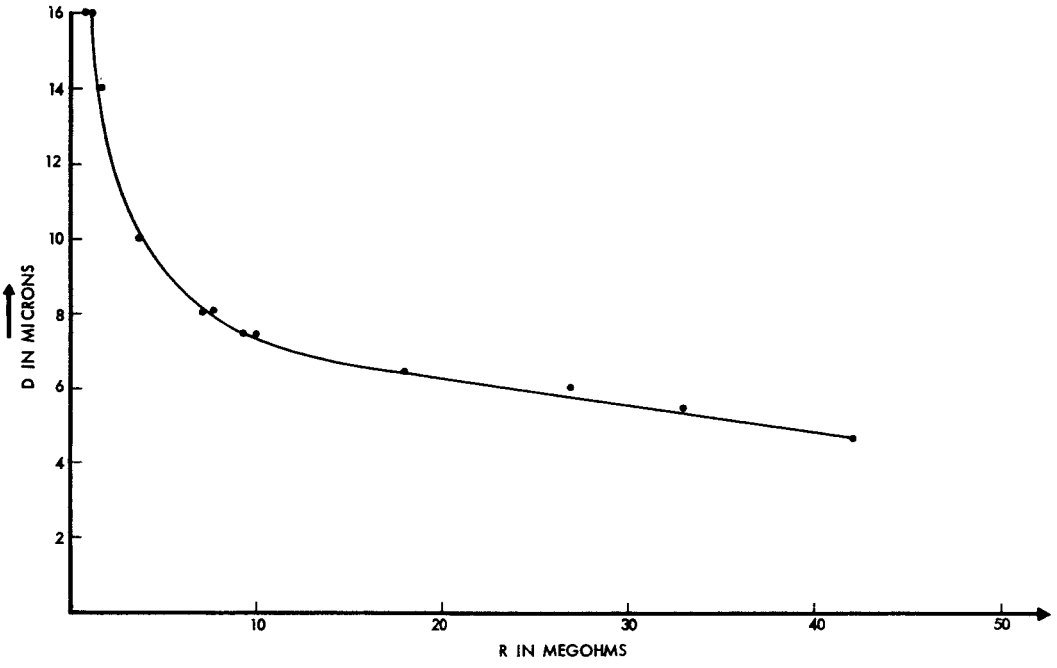

FIGURE 1. - IMPEDANCE LEVEL OF GLASS MICROELECTRODES FILLED WITH 0.9% NaCl. INNER TIP DIAMETER D IN MICRONS PLOTTED VERSUS ELECTRODE RESISTANCE R IN MEGOHMS. MEASUREMENTS WERE MADE IN NORMAL SALINE USING A BRIDGE CIRCUIT AT A FREQUENCY OF 1,000 CPS.
(App.)

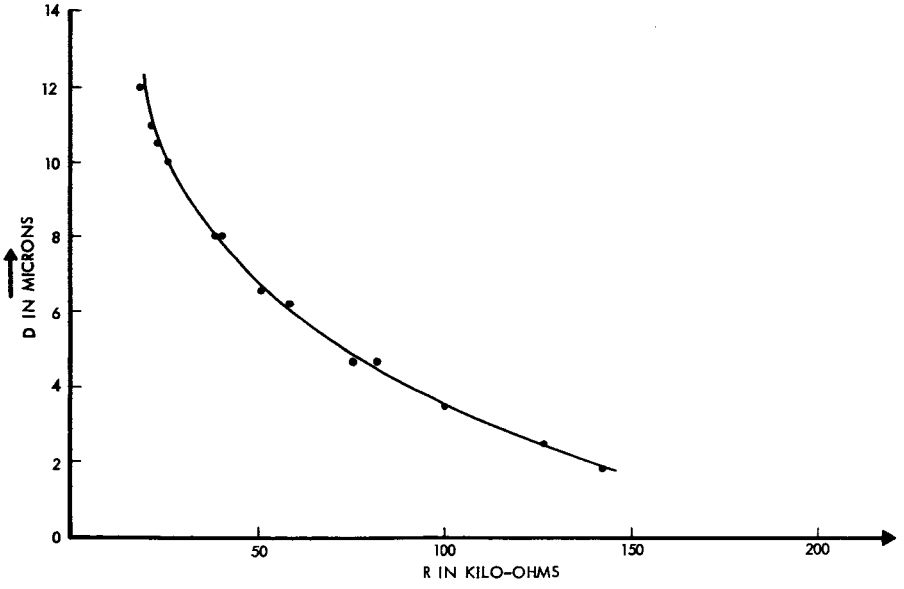

FIGURE 2. - IMPEDANCE LEVEL OF METAL-FILLED MICROELECTRODES WITH A PLATINUM BLACK TIP. DIAMETER D IN MICRONS PLOTTED VERSUS ELECTRODE RESISTANCE R IN KILO-OHMS. TECHNIQUE OF MEASUREMENT AS IN FIGURE 1App.
(App.)

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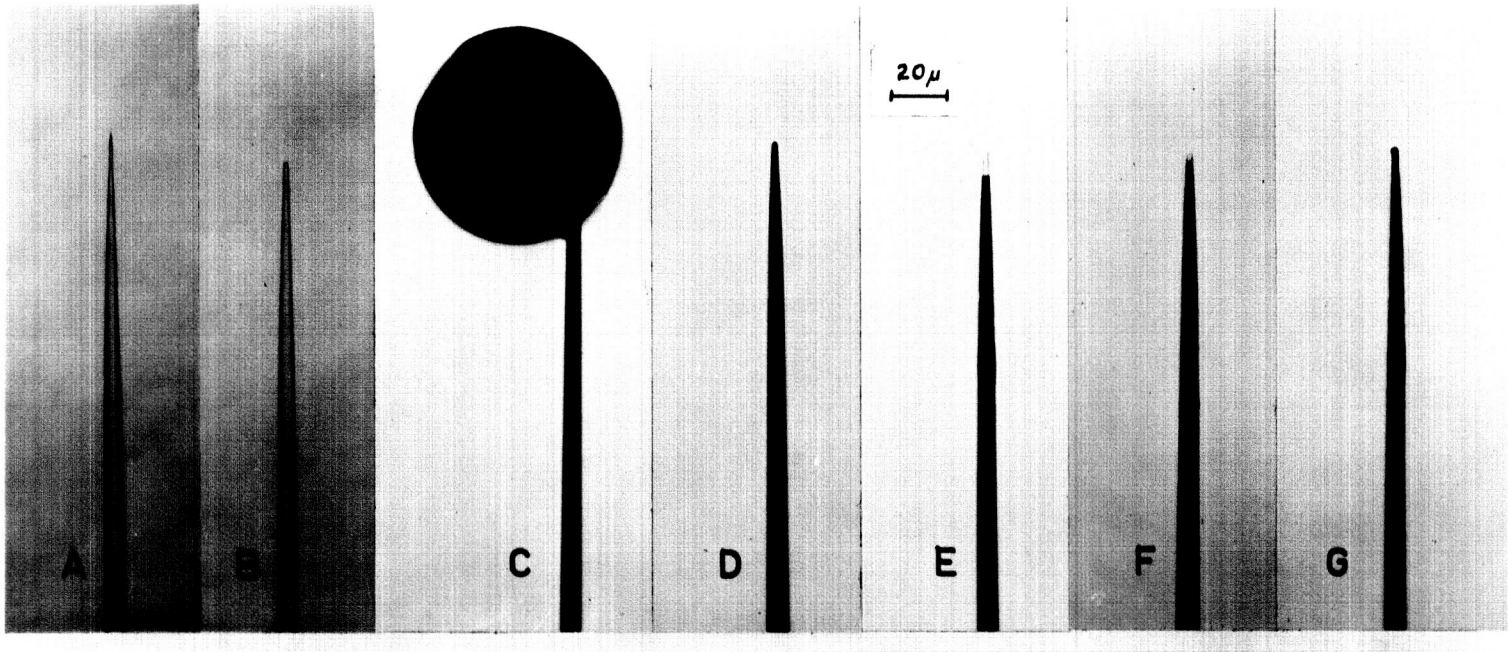

Fig. 3 app.: Microphotographs demonstrating seven stages in preparation of metal-filled microelectrodes. The distal 170 μ of the electrode are shown. The inner tip diameters of B through G are 2 μ.

A. Unfilled hyperfine glass tip of the microelectrode drawn from a point approximately 3 to 4 mm beyond the indium column.

B. Micro glass tip of 2 μ inner tip diameter. Either drawn directly or obtained by careful breakage of a hyperfine glass tip (A) under microscopic inspection.

C. After repeated heating on a hot plate in a vertical position the indium column has filled the pipette completely and formed a tiny drop at the tip.

D. The tiny metal ball has been blown off, leaving the completely filled micropipette.

E. Approximately 8 μ of the indium core has been electrolytically dissolved in a solution of 20 % ferric nitrate.

F. Silver has been electrolytically positioned on end of indium core to within approximately 3 μ of the electrode tip.

G. Platinum and platinum black have been sequentially deposited, forming a tiny knob-like end.

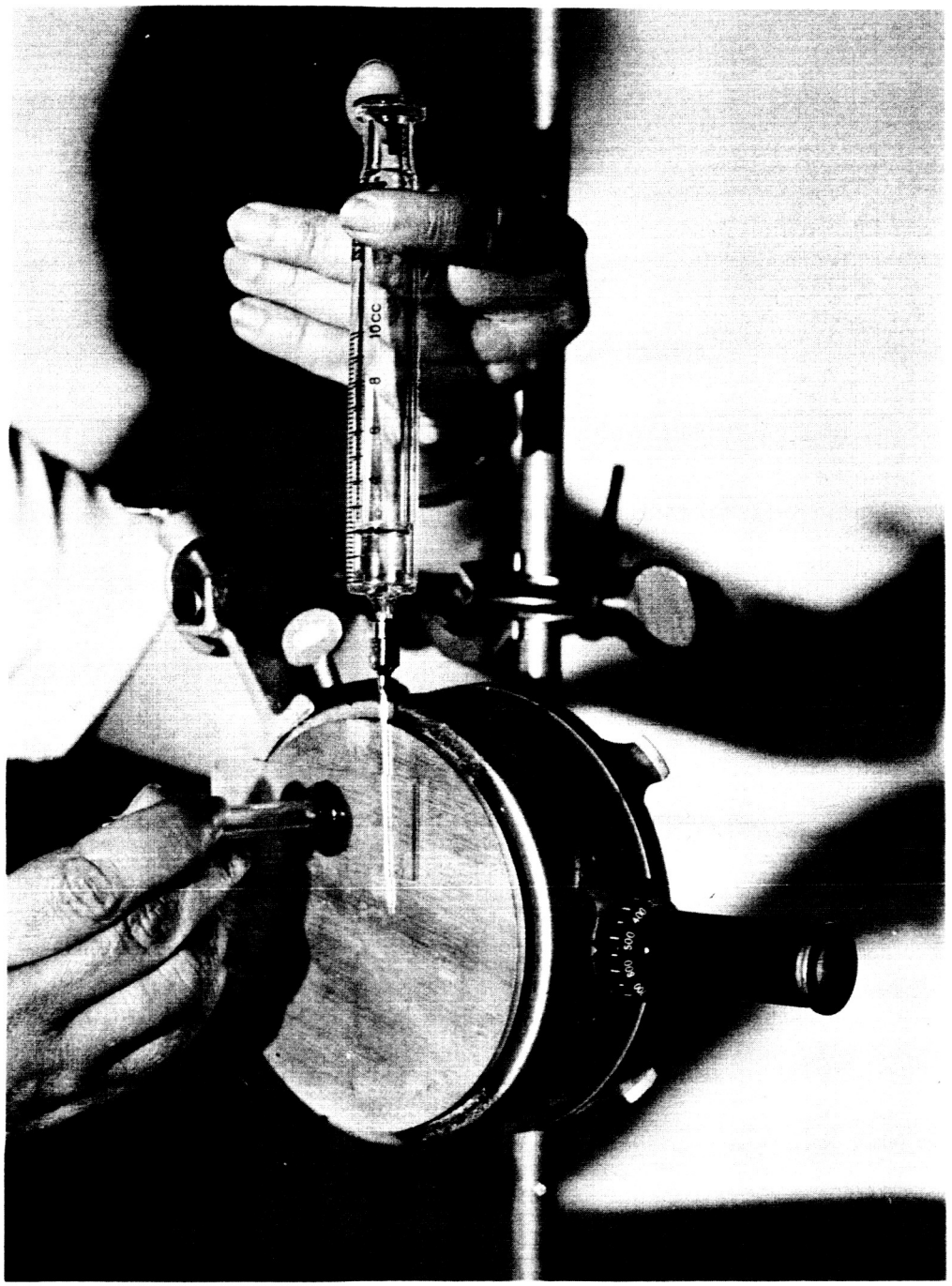

Figure 4 app. Glass micropipette is held to a hot plate in a vertical position for filling procedure with indium. For details see text.

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